REMARKS

Applicant's Summary of Telephonic Interview

The undersigned wishes to thank Examiner Betton and SPE Sreeni Padmanabhan for the courtesy of extending a telephonic interview to the undersigned on Thursday, June 10, 2010. Elected claim 19 under consideraiton at the interview read as follows:

> Claim 19 (**previously presented**): A method of treating or reducing the extent of atherosclerosis in a warm-blooded animal in need thereof, which comprises administering to said animal an effective amount of a combination comprising candesartan or a pharmaceutically acceptable salt thereof and rosuvastatin or a pharmaceutically acceptable salt thereof.

The rejection of elected claim 19 as being obvious over the Robl reference was discussed, together with Applicant's position expressed in its August 27, 2009 response, that the remote chance a skilled person might select the specfically claimed combination of candesartan and rosuvastatin from the vast number of alternatives listed in Robl did not meet even the obvious-to-try criteria of the Supreme Count opinion in *KSR*. Nevertheless, as an expediency to accelerate the examination of this application to allowance, the undersigned proposed to narrow the transitional phrase "effective amount of a combination *comprising*..." to read "effective amount of a combination *consisting essentially of*...*as the sole therapeutically effective agents*...." The undersigned further pointed out that that a claim so narrowed would have to also specifically encompass the additional presence of a diuretic in that a combination of candesartan and rosuvastatin optionally including a diuretic (specifically hydrochlorothiazide) is taught in the specification. It was understood from the interview that amending claim 19 in this manner (as has been done by the above amendments) would be favorably considered.

However Examiner Padmanabhan also expressed the view that the claims should be limited to the specific doses used in the example. The undersigned pointed out that since the comparative testing in the example was carried out in mice based on an accepted animal model for atherosclerosis, limiting the claims to the mouse dose levels was neither practical nor required under the case law, such as the Federal Circuit decision in *In re Brana*, 34 USPQ2d 1436 (Fed. Cir. 1995). The interview ended with the suggestion that the above claim

amendments be made, and that arguments respecting the dose level would be considered. Otherwise, no agreement was reached.

Subsequent to the interview the undersigned determined that the above amendments and the more comprehensive comments and arguments presented below could more effectively be presented in this Amendment and Response Accompanying RCE than in a response after final rejection. It is therefore respectfully requested that the above amendments be entered and that the following arguments be given full and careful consideration relative to the outstanding rejection over the Robl reference.

Claim Amendments

As proposed and discussed at the interview, claim 19 is amended above as follows:

Claim 19 (**currently amended**): A method of treating or reducing the extent of atherosclerosis in a warm-blooded animal in need thereof, which comprises administering to said animal an effective amount of a combination ~~comprising~~ consisting essentially of candesartan or a pharmaceutically acceptable salt thereof and rosuvastatin or a pharmaceutically acceptable salt thereof as the sole therapeutically effective agents, and optionally a diuretic which is hydrochlorothiazide.

The optional inclusion of a diuretic, and specifically hydrochlorothiazide, is specifically disclosed in the specification at page 2, lines 19-21.

New claim 27 provides for the method of claim 19 wherein the composition is in the form of a single oral formulation, having specification support, *e.g.*, at page 4, lines 30-32.

New claim 28 provides for the method of claim 19 or claim 27 wherein the composition is administered in association with a pharmaceutically acceptable diluent or carrier, having specification support at page 3, lines 14-17 and in the paragraph bridging pages 3 and 4.

New claims 29 and 30 provide for the method of claim 19, claim 27 or claim 28 wherein candesartan is administered in the form of candesartan cilexetil, having specification support, *e.g.*, at page 2, lines 19-20.

It should be clear from the above that no new matter has been added by the above amendments. These amendments are being made without waiver or prejudice to Applicant's right to prosecute any subject matter thereby deleted in one or more divisional or continuing applications.

Following entry of these amendments, claims 19 and 27-30 are pending in this application.

Claim Rejections under 35 U.S.C. § 103

Claim 19 is rejected under 35 U.S.C. 103(a) as being unpatentable over Robl, US Patent 6,620,821(hereinafter "Robl '821") alone. This ground for rejection is respectfully traversed in view of the above amendments and the following remarks.

Applicant's Invention as Presently Claimed is Not Rendered Prima Facie Obvious by the Robl Disclosure

The invention of Robl '821 is a new HMG CoA reductase inhibitor most generally referred to and illustrated therein as compounds of "structure I." Compounds of structure I clearly do not encompass either rosuvastatin or candesartan. Other aspects of the invention are the administration of compounds of structure I for the treatment of a variety of disease conditions listed over columns 5 and 6 of the specification, the list including "preventing or reversing progression of atherosclerosis" along with myriads of other diverse disease conditions.

Following the description of the compounds of Robl's invention and the disease conditions treated thereby, Robl '821 notes in the middle of column 28 that the "HMG CoA reductase inhibitors of formula I may be employed in combination with *all therapeutic agents* which are useful in combination with HMG CoA reductase inhibitors," with no indication of the objective to be achieved by making such combinations, or even an indication as to why these other therapeutic agents "are useful in combination with HMG-CoA reductase inhibitors." This is followed by an extensive lising of the various different *types* of other therapeutic agents with which compounds of Robl's structure I may be combined, which listing is followed by a further *12 column list* of possible therapeutic agents that may be administered with Robl's compounds of structure I. Within this vast compilation of diverse therapeutic agents is rosuvastatin listed at column 29, line 39-40, and candesartan, *separatedly listed eight columns later*, at column 37, line 19.

Applicant previously argued that this separate, unassociated listing of rosuvastatin and candesartan, eight columns apart in this 12 column exhaustive recitation of myriads of other diverse types of therapeutic agents, one or more of which may be used in combination with the

compounds of Robl's structure I, does not constitute "*a finite number* of identified, predictable solutions" required by the Supreme Court in *KSR*[1] to make the combination of candesartan with rosuvastatin "obvious to try" in the treatment of atherosclerosis. Applicant further pointed out that there is *no particular objective or goal* stated in Robl '821 for combining these other therapeutic agents with compounds of Robl's structure I. Thus there also is no "*design need or market pressure to solve a problem*" with *a finite number of identified, predictable solutions*, as is also required by *KSR* for this reference to make a combination of candesartan with rosuvastatin "obvious to try." Accordingly, the disclosure of Robl does not support a finding of *prima facie* obviousness.

However, it is understood in the current Action at pages 2-3 the Examiner is arguing that because of the open "comprising" language in claim 19, applicant is not claiming a "distinct combination featureing exclusively candesartan and rosuvastatin." The Examiner further observes that "one of skill would reasonably interpret claim 19 to importantly contain candesartan and rosuvastatin among other such classes of agents utilized for treatment for the same general disorders," and that "spectrum therapy is well-known" for complex athrosclerotic disorders.

Applicant still maintains that the Robl disclosure does not meet the requirements of the *KSR* Supreme Court decision for even "obvious to try", and therefore Robl does not render claim 19 *prima facie* obvious. Nevertheless, in order to accelerate the prosecution of this application to allowance, the transitional pharse "combination consisting essentially of …" in claim 19 has been amended above to more particularly recite "consisting essentially of candesartan … and rosuvastatin … as the sole therapeutically active agents … ." Because of the more limiting nature of this recitaiton, the claim further provides "and optionally a diuretic which is hydrochlorohydrazide," the optional inclusion of which is clearly a part of the disclosed invention (see, *e.g.*, specification at page 2).

Although no agreement was reached during the June 11, 2010 interview, it is understood that this amendment to the transitional pharse would be favorably considered with respect to the overcoming *prima facie* obviousness rejection.

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[1] *KSR International Co. v. Teleflex Inc.*, 127 S. Ct. 1727 [82 USPQ2d 1385, 1398] (2007).

In this regard, the Examiner is respectfully reminded that while *KSR* may be considered to have eased the showings required for *prima facie* obviousness, it has not removed all requirements. The Court in *KSR* explained that the Federal Circuit's "teaching, suggestion or motivation" test provides helpful insight into the obviousness question as long as it is not applied rigidly and that, accordingly, it remains necessary for the Examiner to *identify some reason* that would have led a chemist to modify the prior art in a particular manner to establish *prima facie* obviousness of the claimed invention. Moreover, "obvious to try" does not arise simply because the components of the claimed invention are separately known in the art, but rather a particular combination might be obvious to try only when "there is a design need or market pressure to solve a problem and there are *a finite number of identified, predictable solutions*, and a person of ordinary skill has good reason to pursue the known options within his or her technical grasp."

The Supreme Court's *KSR* reasoning was summarized and applied by the Federal Circuit, for example, in its recent decision in *Procter & Gamble Co. v. Teva Pharmaceuticals USA Inc.*, 90 USPQ2d 1947, 1949-50 (Fed. Cir. 2009). After noting that the obviousness determination turns on the four underlying Graham v. John Deere factual inquiries, the Court continued:

> The Supreme Court has explained that *the Federal Circuit's "teaching, suggestion or motivation" test provides helpful insight into the obviousness question as long as it is not applied rigidly*. KSR Int'l Co. v. Teleflex Inc., 550 U.S. 398, 127 S. Ct. 1727, 1741 [82 USPQ2d 1385] (2007). Accordingly, under KSR, "*it remains necessary to identify some reason that would have led a chemist to modify a known compound in a particular manner to establish prima facie obviousness of a new claimed compound.*" Takeda Chem. Indus., Ltd. v. Alphapharm Pty., Ltd., 492 F.3d 1350, 1357 [83 USPQ2d 1169] (Fed. Cir. 2007).

(90 USPQ2d at 1949-50; emphasis added). The Court continued:

> When a person of ordinary skill is faced with "a finite number of identified, predictable solutions" to a problem and pursues "the known options within his or her technical grasp," the resulting discovery "is likely the product not of innovation but of ordinary skill and common sense." *KSR*, 127 S. Ct. at 1742. So too, "[g]ranting patent protection to advances that would occur in the ordinary course without real innovation retards progress." *Id.* at 1741. *In other cases,* though, researchers can only "vary all parameters or *try each of numerous possible choices until one possibly arrive[s] at a successful result, where the prior art [gives] either no indication of which parameters*

> *[are] critical or no direction as to which of many possible choices is likely to be successful.*" *In re O'Farrell*, 853 F.2d 894, 903 [7 USPQ2d 1673] (Fed. Cir. 1988). In such cases, "*courts should not succumb to hindsight claims of obviousness.*" *In re Kubin*, 561 F.3d 1351, No. 2008-1184, slip op. at 14 [90 USPQ2d 1417] (Fed. Cir. Apr. 3, 2009).

(90 USPQ2d at 1952; emphasis added),

It is respectfully submitted that Robl does not give the skilled person a finite number of identified, predictable solutions to a problem, and certainly gives no guidance or motivation to combine candesartan with rosuvastatin, and optionally hydroclorothiazide as the sole therapeutically active agents as now claimed. Accordingly, even under *KSR* and its more flexible "obvious to try" threshold, the specifically defined combination of the presently amended claims clearly is not rendered *prima facie* obviousness by Robl '821, as the Federal Circuit has interpreted and applied *KSR* to pharmaceutical inventions.

Limiting the Claims to the Mouse Dose Levels Use in the Acceped Animal Model of the Example is Neither Legally Required nor Practical

As noted above, SPE Padmanabhan raised a further question during the interview as to whether claim 19 should also recite the specific dose of 1 mg/kg/day for both candesartan and rosuvastatin at which synergistic results were demonstrated in the animal model (mice) used in the comparative testing set forth and discussed at pages pages 7-11 of the specification and illustrated in Figures 1 and 2. However, it is not clear whether the request was made in the legal context of enablement or in the context of demonstrating unexpected results to overcome *prima facie* obviousness, if still needed.

If the Examiner's request that specific mouse dose levels be put in the claims is from the perspective of enablement, it is respectfully submitted that the specification provides sufficient guidance to persons skilled in the art to determine the appropriate dose levels, *e.g.,* for use in human subjects. For example, the Examiner's attention is called to the specification disclosure at page 4, lines 9 through 26. As will be discussed further below, it is not a requirement for patentability that human testing in clinical trials be conducted. Moreover, the fact that clinical trials in humans have been commenced is strong indication that persons skilled in the art are sufficiently confident of the success of the combination that the initiation of time consuming and costly clinical trials is merited. In fact, one purpose of clinical testing following animal testing is

to determine the most appropriate dose for human subjects, *e.g.*, through the common practice of dose titration. Certainly dose titration to determine appropriate dose levels in humans cannot be considered to be "undue experimentation." Accordingly, it is respectfully submitted the amended claims are fully enabled by the dose guidance provided in the the specification, particularly considering that dose titration is routinely used in the pharmaceutical industry to determine appropriate dose levels.

On the other hand, if the Examiner's request that the mouse dose levels be inserted in the claims is from the persepctive of demonstrating unexpected results to overcome a finding of *prima facie* obviousness, it is believed that Applicant has avoided or obviated any finding of *prima facie* obviousness over Robl by the above claim amendments and the foregoing remarks. Thus, there *should be no need for Applicant to demonstrate unexpected or syergistic results* from the invention as claimed since there is no *prima facie* obviousness to be overcome.

Neverthtless, *even if* the Examiner maintains that the claims as now amended are *prima facie* obvious over Robl '821, it is respectfully submitted that the unexpected results demonstrated by the comparative evidence based on an accepted animal model is sufficient to overcome any such *prima facie* obviousness, without need to incorporate in the claims the mouse dose levels that were used.

Even If One Assumes That Prima Facie Obviousness Has Been Established, Any Such Prima Facie Obviousness Has Been Overcome By The Synergystic Results Demonstrated By The Comparative Accepted Animal Model Data Presented

Even if it is assumed that a case of *prima facie* obviousness has somehow been made, the undersigned pointed out during the interview that requiring inclusion in the claims of the specific mouse dose used in the testing is unrealistic since the claims clearly are not limited to the treatment of atherosclerosis in mice. Such a requirement is contrary to the established case law that endorses the sufficiency of accepted animal models to support issues of patentability, and which also makes very clear that it is impractical and erroneous for the Patent Office to require evidemce from clinical testing as a condiiton to patentability. Therefore, any assertion of *prima facie* obviousness that might be made of the present claims is overcome by the comparative data clearly demonstrating synertistic results, which data was generated using an art accepted animal model as set forth in the specification.

Thus, it is respectfully submitted that it is error for the Examiner to require Applicant to limit its claims to dose levels *suitable for experimental mice*. In practical effect, this would preclude Applicant from establishing patentability of its method of treatment claims by means of data obtained using an art accepted animal model, and thus *require that Applicant conduct human clinical trials* in order to obtain a claim scope that would encompass human subjects.

Such a requirement is directly contrary to the Federal Circuit decision in *In re Brana*, 34 USPQ2d 1436 (Fed. Cir. 1995), which expressly sanctions the use of statistically significant data from art accepted animal models for purposes of patentability (as opposed to requiring human clinical data).

(1) Acceptance of Animal Data for Patentability Purposes

The acceptance of animal data (as opposed to requiring clinical trials) *for patentability purposes* is made very clear in the Federal Circuit decision of *In re Brana*, 34 USPQ2d 1436 (Fed. Cir. 1995) (copy attached for the Examiner's convenience). Particular note should be taken from the following discussion *that the Federal Circuit took pragmatic approach in applying the tests and requirements for patentability*, recognizing the realities and timing faced by inventors trying to obtain meaningful patent coverage of pharmaceutical inventions. The Court specifically rejected the Commissioner's requirement, in effect, for Phase II human clinical data noting that the associated costs "would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer" (34 USPQ2d at 1442-43; emphasis added).

The claims there in issue were directed toward compounds which were said to have "a better action and better action spectrum as anti-tumor substances" than previously published compounds. The previously published compounds had been screened for anti-tumor activity by testing their efficacy *in vivo* against two implanted leukemias in a mouse model. These *in vivo* tests were widely used by the National Cancer Institute to measure the anti-tumor properties of a compound. Applicant's specification, however, only illustrated the cytotoxicity of the claimed compounds against human tumor cells *in vitro*, and concluded that these tests "had a good action."

There initially was a rejection for *prima facie* obviousness under §103, which applicant rebutted by asserting unexpectedly better anti-tumor properties, including a declaration reporting tests *in vitro,* which were said to indicate that applicants' claimed compounds were far more effective as antitumor agents than the compounds disclosed in the prior art, using two specific types of human tumor cells. It is noteworthy that these animal tests were deemed sufficient to overcome the §103 rejection. However, the Examiner nevertheless issued a final rejection for non-enablement under §112, ¶ 1, asserting, *inter alia*, that the prior art tests of the previous publication and the tests disclosed in the specification were not sufficient to establish a reasonable expectation that the claimed compounds had practical utility. While the final rejection, the Board affirmance thereof and the Federal Circuit decision all addressed the rejection *as a non-enablement rejection under §112*, the underlying issue, as in the present case, was the use of an acceptable animal model to establish efficacy in a human subject.

The Court noted that applicants provided test results through a declaration, showing that several compounds within the scope of the claims exhibited significant antitumour activity against the L1210 standard tumor model *in vivo*, which "evidence alone should have been sufficient to satisfy applicants' burden" (34 USPQ2d at 1441-42). The Court continued:

> The Commissioner counters that such *in vivo* tests in animals are only preclinical tests to determine whether a compound is suitable for processing in the second stage of testing, by which he apparently means *in vivo* testing in humans, and therefore are not reasonably predictive of the success of the claimed compounds for treating cancer in humans. The Commissioner, as did the Board, confuses the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption. See *Scott v. Finney*, 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120 (Fed.Cir. 1994) ("Testing for the full safety and efficacy of a prosthetic device is more properly left to the Food and Drug Administration (FDA). Title 35 does not demand that such human testing occur within the confines of Patent and Trademark Office (PTO) proceedings.").
>
> Our court's predecessor has determined that proof of an alleged pharmaceutical property for a compound by statistically significant tests with standard experimental animals is sufficient to establish utility. *In re Krimmel*, 292 F.2d 948, 953, 130 USPQ 215, 219 (CCPA 1961); see also *In re Bergel*, 292 F.2d 958, 130 USPQ 205 (CCPA 1961). In concluding that similar in *vivo tests* were adequate proof of utility the court in *In re Krimmel* stated:
>
> > We hold as we do because it is our firm conviction that one who has taught the public that a compound exhibits some desirable

> pharmaceutical property in a standard experimental animal has made a significant and useful contribution to the art, even though it may eventually appear that the compound is without value in the treatment of humans.
>
> *Krimmel*, 292 F.2d at 953, 130 USPQ at 219. Moreover, NCI apparently believes these tests are statistically significant because it has explicitly recognized both the P388 and L1210 murine tumor models as a standard screening test for determining whether new compounds may be useful as antitumour agents.

(34 USPQ2d at 1442; emphasis added).

The Commissioner had cited two literature references (Martin and Pazdur) for the proposition that laboratory oncologists are skeptical about the predictive value of *in vivo* murine tumor models for human therapy. However, the Court dismissed this assertion, noting that even Martin recognizes that these tumor models continue to contribute to an increasing human cure rate. The Court then continued:

> On the basis of animal studies, and controlled testing in a limited number of humans (referred to as Phase I testing), the Food and Drug Administration may authorize Phase II clinical studies. See 21 U.S.C. Section 355(i)(1); 5 C.F.R. Section 312.23 (a)(5), (a)(8) (1994). Authorization for a Phase II study means that the drug may be administered to a larger number of humans, but still under strictly supervised conditions. The purpose of the Phase II study is to determine primarily the safety of the drug when administered to a larger human population, as well as its potential efficacy under different dosage regimes. See 21 C.F.R. Section 312.21(b).
>
> FDA approval, however, is not a prerequisite for finding a compound useful within the meaning of the patent laws. *Scott*, 34 F. 3d 1058, 1063, 32 USPQ2d 1115, 1120. Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we do require Phase II testing in order to prove utility the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer.
>
> In view of all of the foregoing, we conclude that applicants disclosure complies with the requirements of 35 U.S.C. Section 112 Para. 1.

(34 USPQ2d at 1442-43; emphasis added).

The current Patent Office Board of Appeals and Interferences has widely followed the pragmatic approach of the Federal Circuit of *In re Brana* (as it must). While *In re Brana* and the

Board decisions discussing *Brana* are generally addressing non-enablement under section 112, the Board has recognized that the pragmatic approach of *Brana* with respect to the sufficiency of animal testing in pharmaceutical cases should be applied to all patentability issues. Thus in *Ex parte Gregory*, Appeal 2008-005266 (BPAI 2009) (copy attached for the Examiner's convenience), the Board noted:

> Moreover, "[w]hen prima facie obviousness is established and evidence is submitted in rebuttal, the decision-maker must start over." *In re Rinehart*, 531 F.2d 1048, 1052 (CCPA 1976); *In re Hedges*, 783 F.2d 1038, 1039 (Fed. Cir. 1986) ("If a prima facie case is made in the first instance, and if the applicant comes forward with reasonable rebuttal, whether buttressed by experiment, prior art references, or argument, the entire merits of the matter are to be reweighed"). Thus, all of the evidence must be considered under the Graham factors in reaching the obviousness determination.
>
> In speaking about the relationship of patent law and FDA law, the Federal Circuit has noted:
>
> > On the basis of animal studies, and controlled testing in a limited number of humans (referred to as Phase I testing), the Food and Drug Administration may authorize Phase II clinical studies. See 21 U.S.C. § 355(i)(1); 5 C.F.R. § 312.23 (a)(5), (a)(8) (1994). Authorization for a Phase II study means that the drug may be administered to a larger number of humans, but still under strictly supervised conditions. The purpose of a Phase II study is to determine primarily the safety of the drug when administered to a larger human population, as well as its potential efficacy under different dosage regimens. See 21 C.F.R. § 312.21(b). FDA approval, however, is not a prerequisite for finding a compound useful within the meaning of the patent laws. . . . . Usefulness in patent law, and in particular the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer.
>
> *In re Brana*, 51 F.3d. 1560, 1568 (Fed. Cir. 1995) (citations omitted). Although the above statements were made in the context of utility and enablement, the clear inference is that FDA determinations are not controlling on patentability, which would include the obviousness determination.

(Ex parte Gregory at pages 8-10; emphasis added).[2]

In view of the above legal analysis, it is respectfully submitted that comparative data generated by the animal model at pages 7-11 and illustrated in Figures 1-2 presents sufficient statistically significant evidence of a synergistic effect in the reduction of atherosclerosis as compared to either component alone.

The "Animal Model" described at page 7 used "apo-E knockout mice." The use of "apo-E knockout mice" (also referred to as Apolipoprotein E deficient mice, or ApoE-deficient ($apoE^{-/-}$) mice) clearly was an art accepted animal model for evaluation of drugs in atherosclerosis prior to and at the time the priority application was filed in 2003, as well as thereafter to the present time. In this regard, the Examiner's attention is called to the four literature references submitted herewith, being Breslow, *Mouse Models of Atherosclerosis*, Science, 1996, 272, 685-688 ("Breslow (1996)"); Harris et al., *Inhibition of atherosclerosis in apolipoprotein-E-deficient mice following muscle transduction with adeno-associated virus vestors encoding numan apolipoprotein-E*, Gene Therapy 2002, 9, 21-29 ("Harris (2002)"); Lyngdorf et al, *Paradoxical reduction of atherosclerosis in apoE-deficient mice with obesity-related type 2 diabetes*, Cardiovascular Research, 2003, 59, 854-862 ("Lyngdorf (2003)"); and Tarling et al., *Impaired Development of Atherosclerosis in $Abcg1^{-/-}$ $Apoe^{-/-}$ Mice,* Arteriosclerosis, Thrombosis and Vascular Biology, 2010, 30, 1174 ("Tarling (2010)"). For example, the Abstract of the Breslow (1996) reference states:

> As a species the mouse is highly resistant to atherosclerosis. However, through induced mutations it has been possible to develop lines of mice that are susceptible to this disease. For example, mice that are deficient in apolipoprotein E, a ligand important in lipoprotein clearance, develop atherosclerotic lesions resembling those observed in humans. These lesions are exacerbated when the mice are fed a high-cholesterol, high-fat, Western-type diet. Other promising models are mice that are deficient in the low density lipoprotein receptor and transgenic mice that express human apolipoprotein B and transdominant mutant forms of apolipoprotein E. These models are now being used to study the pathogenesis of atheroclerotic lesions, as well as the influence of genetics, environment, hormones, and drugs on lesion development.

[2] The facts of *In re Gregory* did not involve animal models. Rather, applicant was claiming a 300mg capsule of trimethobenzamide, which fell within the range of the 100mg, 200mg, 250mg and 400mg capsules previously approved by the FDA. The Board rejected applicant's assertion that FDA approval of its 300mg capsule demonstrated criticality sufficient to overcome *prima facie* obviousness.

(Breslow (1996) at page 685; emphasis added). *See also* Harris (2002), which states at the top of page 22, "The apoE$^{-/-}$ transgenic mouse has been used extensively as an animal model for atherosclerosis as it develops severe hypercholestrolaemia and atherosclerotic lesions similar to thos found in humans."

As reported under "Results" at page 10 of the present application, candesartan and rosuvastatin alone decreased atherosclerosis by about 35% and 25% respectively, whereas the combination reudced atherosclerosis by 70%, demonstrating a synergistic effect, which effect is illustrated graphically in Figure 2.

Thus, clearly Applicant has demonstrated by means of an art-accepted animal model a synergistic, unexpected effect of the claimed combination in decreasing atherosclerosis relative to each component alone.

Moreover, the specification discloses appropriate dose ranges and routes of administration for the components of the claimed composition in a manner sufficient to meet the enablement requirements of section 112. While the Examiner has not suggested that the present claims are not enabled, for completeness of the present analysis, it is pointed out at page 4 of the specification that rosuvastatin and candesartan are both commercially available and appropriate dosage ranges and routes of administration are disclosed at pages 4-5. Similar type dosage information in a specification has been found adequately enabling. For example, the following specification dose information was found enabling, to "adequately convey to any person skilled in the art useful daily dosage information for the claimed compounds" in *Ex parte Porubek*, Appeal No. 2001-1101 (BPAI, non precedential) (copy attached for the Examiner's convenience):

> While dosage values will vary, therapeutic compounds of the invention may be administered to a human subject requiring such treatment as an effective oral dose of about 50 mg to about 5000 mg per day, depending upon the weight of the patient. For any particular subject, specific dosage regimens should be adjusted to the individual's need and to the professional judgment of the person administering or supervising the administration of the inventive compounds.

(Appeal 2001-1101 at page 5).

The present disclosure with respect to dose ranges and routes of administration is far more informative, and it is respectfully submitted fully meets the requirements of the patent laws.

Update on Applicant's PCT and Corresponding European Application

Applicant noted in the August 27, 2009 Amendment and Reply at page 13 that the corresponding European Application EP04768663 had been allowed and was about to grant. To complete the record, a copy of granted European Patent EP1673091, a copy of which is provided with the Supplemental Information Disclosure Statement submitted herewith.

Information Disclosure Statement

The Examiner's attention respectfully drawn to the further Information Disclosure Statement submitted herewith, in which are cited additional documents which have been brought to Applicant's attention during the course of prosecution of corresponding foreign applications. These documents are listed on the form PTO-1449 submitted therewith, and copies the cited documents (other than US patents and published applications) are being provided.

Conclusion

All outstanding grounds for rejection have been addressed and, it is believed, overcome by the above amendments, the foregoing remarks and the attached supporting literature references. It is therefore respectfully submitted that all claims are now in condition for allowance, and a Notice to that effect is respectfully requested.

EXCEPT for issue fees payable under 37 C.F.R. § 1.18, the Director is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. §§ 1.16 and 1.17 which may be required, including any required extension of time fees, or credit any overpayment to Deposit

Account 50-0310. This paragraph is intended to be a **CONSTRUCTIVE PETITION FOR EXTENSION OF TIME** in accordance with 37 C.F.R. § 1.136(a)(3).

Respectfully Submitted,
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assess risk in familial cases where the specific mutation is not yet known.

Unfortunately, sensitive genetic testing of genetically heterogeneous disorders is not yet practical for patients who are not members of families with a defined mutation. For example, mutations in at least five different genes can cause LQT, yet only three of these genes have been identified. Moreover, 40 mutations have already been defined in the three known genes. Analysis of risk in an individual who is not part of an LQT family would require mutational analysis of all LQT-causing genes. Advances in the sensitivity and efficiency of genetic testing, coupled with continued molecular genetic discoveries, will enable more reliable and cost-effective analysis of risk for cardiovascular disorders in the general population.

Risk stratification is another important application of molecular genetics. In FHC, for example, certain mutations of the gene encoding β cardiac myosin heavy chain carry substantially greater risk of sudden death (32). Substitution of Glu for Gly at position 256 is associated with a disease penetrance of only 56% and a benign prognosis, whereas substitution of a Gln for Arg at position 403 is associated with 100% disease penetrance and a high risk of sudden death (33). Similarly, physiologic studies of LQT-associated mutations in HERG indicate a spectrum of HERG K+ channel dysfunction, which ranges from a partial loss of function to complete dominant negative suppression. Although the power of a single piece of genetic information can be limited by modification of genetic and environmental factors, this prognostic information can nevertheless be quite useful in inherited cardiovascular disorders. This is particularly true when one can select from a spectrum of therapies that are increasingly aggressive, such as medical therapy versus implantation of an internal defibrillator for FHC or LQT.

Thus, in less than a decade, the techniques of molecular genetics have contributed dramatically to our understanding of cardiovascular disease pathogenesis. Many genes that have a major effect on cardiovascular risk have already been identified, and genetic diagnosis, prognosis, and mechanism-based therapy are available in some cases. Continued genetic discoveries and technological advances are likely to make genetic testing and genotype-based therapy a routine part of clinical care in the future.

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Mouse Models of Atherosclerosis

Jan L. Breslow

As a species the mouse is highly resistant to atherosclerosis. However, through induced mutations it has been possible to develop lines of mice that are susceptible to this disease. For example, mice that are deficient in apolipoprotein E, a ligand important in lipoprotein clearance, develop atherosclerotic lesions resembling those observed in humans. These lesions are exacerbated when the mice are fed a high-cholesterol, high-fat, Western-type diet. Other promising models are mice that are deficient in the low density lipoprotein receptor and transgenic mice that express human apolipoprotein B and transdominant mutant forms of apolipoprotein E. These models are now being used to study the pathogenesis of atherosclerotic lesions, as well as the influence of genetics, environment, hormones, and drugs on lesion development.

Atherosclerotic cardiovascular disease is the major cause of morbidity and mortality in much of the world. Atherogenesis is a complex process in which the lumen of a blood vessel becomes narrowed by cellular and extracellular substances to the point of obstruction. Lesions tend to form at the branch points of arterial blood vessels and progress through three stages (Fig. 1). The first stage is the fatty streak lesion, which is characterized by the presence of lipid-filled macrophages (foam cells) in the subendothelial space. The second stage is the fibrous plaque, which consists of a central acellular area of lipid, derived from necrotic foam cells, covered by a fibrous cap containing smooth muscle cells and collagen. The final stage is the complex lesion, which shows evidence of thrombus formation with deposition of fibrin and platelets.

Researchers in vascular biology are

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working to identify the important cells and molecules involved in each stage of atherogenesis, as well as the environmental and genetic factors that promote lesion formation. These are complex questions that require in vivo models that mimic the human disease. Experimental approaches that deviate widely from the human disease or rely too heavily on in vitro systems could be misleading. Until recently, atherogenesis had been studied mainly in primates and in low density lipoprotein (LDL) receptor-deficient rabbits. Unfortunately, these systems cannot provide sufficiently large numbers of animals, nor do they lend themselves to genetic analysis.

Development of Mouse Models

About 10 years ago, several laboratories attempted to produce atherosclerosis in mice in order to identify potential modifier genes. Mice are highly resistant to atherosclerosis. On a low-cholesterol, low-fat diet, they typically have cholesterol levels of <100 mg/dl, mostly contained in the antiatherogenic high density lipoprotein (HDL) fraction, and do not develop lesions. However, when mice were fed a very high cholesterol, high-fat diet that also contained cholic acid, their cholesterol levels rose by a factor of two to three, with the majority now in the non-HDL fraction. After many months on this diet, certain inbred strains of mice, such as C57BL/6, developed several layers of foam cells in the subendothelial space in a circumscribed area near the aortic valve leaflets, whereas other inbred strains, such as C3H/HeJ, did not (*1*). Crosses between susceptible and resistant strains were used to identify potential genetic susceptibility loci. The most carefully studied of these loci, *ath1*, was mapped to chromosome 1 in the region of the gene that codes for the second most abundant HDL apolipoprotein, apolipoprotein A-II (apoA-II) (*2*).

Although initially promising, the model had two problems. First, in contrast to human lesions, which occur at branch points of major vessels and progress to the fibrous plaque stage, the mouse lesions were small, occurred only in the region of the aortic valve leaflets, and did not progress. Second, the diet required to produce the lesions was unphysiological, as it contained 10 to 20 times the cholesterol of a Western-type diet plus the unnatural dietary constituent cholic acid. This diet caused a chronic inflammatory state in the atherosclerosis-susceptible C57BL/6 strain but not in the atherosclerosis-resistant C3H/HeJ strain (*3*), which raised the possibility that genetic differences between the strains might relate to diet-induced inflammation rather than to atherosclerosis. This is a valid concern, because diet-induced perturbation of the inflammatory process could obscure the more subtle interplay of immune cells and cytokines involved in atherogenesis.

In 1992, two laboratories used gene knock-out technology to generate mice deficient in apolipoprotein E (apoE) (*4*). ApoE, which is made primarily in the liver, is a surface constituent of lipoprotein particles and a ligand for lipoprotein recognition and clearance by lipoprotein receptors. ApoE-deficient mice have delayed clearance of lipoproteins, and on a low-cholesterol, low-fat diet, their cholesterol levels reach 400 to 600 mg/dl as a result of accumulation of chylomicron and very low density lipoprotein (VLDL) remnants enriched in esterified and free cholesterol (*5*). Notably, these mice develop not only fatty streaks but also widespread fibrous plaque lesions at vascular sites typically affected in human atherosclerosis (*6*, *7*). Lesions form at the base of the aorta and the lesser curvature of the thoracic aorta; at the branch points of the carotid, intercostal, mesenteric, renal, and iliac arteries; and in the proximal coronary, carotid, femoral, subclavian, and brachiocephalic arteries. Lesions begin at 5 to 6 weeks of age with monocyte attachment to the endothelium in lesion-prone areas and transendothelial migration. Fatty streak lesions begin to appear at 10 weeks, and intermediate lesions containing foam cells and spindle-shaped smooth muscle cells appear at 15 weeks. Fibrous plaques appear after 20 weeks; these consist of a necrotic core covered by a fibrous cap of smooth muscle cells surrounded by elastic fibers and collagen. In older mice, fibrous plaques progress. In some advanced lesions there is partial destruction of underlying medial cells with occasional aneurysm formation, and in others calcification occurs in the fibrous tissue. Extensive fibroproliferation can narrow the lumen, even to the point of occlusion of vessels. Complicated lesions characterized by thrombosis have not been found.

One of the hallmarks of atherosclerosis is its exacerbation by high-cholesterol, high-fat diets. This effect is mimicked in apoE-deficient mice (*6*). When these mice were fed a Western-type diet (containing 0.15% cholesterol and 21% fat, derived mainly from milk fat), their cholesterol levels rose to three to four times the levels on the low-cholesterol, low-fat diet, and their lesions increased in size and rate of progression.

Additional mouse models of atherosclerosis have been created by introducing other mutations that also alter lipoprotein profiles. In type III hyperlipoproteinemia, a human dyslipidemia associated with atherosclerosis, patients have mutant forms of apoE that interfere with normal clearance of chylomicron and VLDL remnant lipoproteins. Transgenic mice have been created that express two of these mutant forms of apoE that act in a transdominant manner, apoE Leiden and apoE R142C (Arg^{142} → Cys) (*8*). ApoE Leiden transgenic mice that were fed a very high cholesterol diet containing cholic acid had cholesterol levels of 1600 to 2000 mg/dl and developed fatty streak and fibrous plaque lesions. Lesions were not observed when the mice were fed a low-cholesterol, low-fat diet. ApoE R142C transgenic mice that were fed the very high cholesterol–cholic acid diet had cholesterol levels of 370 mg/dl and developed lesions that were mainly of the fatty streak variety. Lesions were not observed when these mice were fed a low-cholesterol, low-fat diet.

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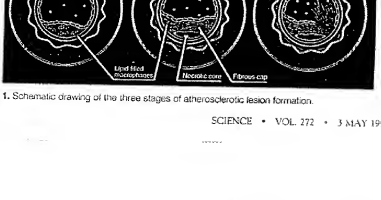

Fig. 1. Schematic drawing of the three stages of atherosclerotic lesion formation.

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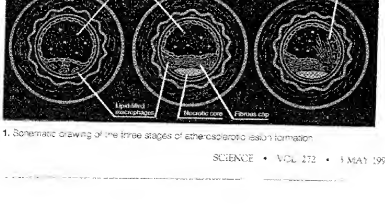

Fig. 1. Schematic drawing of the three stages of atherosclerotic lesion formation.

plasma cholesterol, with most of the increase in the IDL and LDL lipoprotein fractions. This perturbation of the lipoprotein system is insufficient to cause atherosclerosis. However, when LDL receptor–deficient mice were fed a very high cholesterol diet containing cholic acid, they developed cholesterol levels of >1500 mg/dl and had massive fatty streak lesions. Lesions consisting of a lipid-filled necrotic core capped with foam cells were also observed, but there was no evidence of fibrous plaques. LDL receptor knockout mice that were fed the Western-type diet developed cholesterol levels of 1200 mg/dl and had lesions that were mainly fatty streaks (*10*).

Transgenic mice expressing the human *apoB* gene (HuBTg mice) have also been developed as an atherosclerosis model (*11*, *12*). ApoB is the sole protein component in LDL and, as noted above, is the ligand for LDL receptor–mediated removal of LDL from the circulation. On a low-cholesterol, low-fat diet, HuBTg mice have cholesterol levels of 100 to 200 mg/dl and do not develop lesions. However, when these mice were fed a very high cholesterol–cholic acid diet, cholesterol levels rose to 300 to 500 mg/dl and fatty streak lesions occurred. Some of the lesions showed cholesterol crystals in a necrotic core, but fibrous caps were not demonstrated.

LDL receptor–deficient mice and HuBTg mice provide interesting alternatives to apoE-deficient mice as models of atherosclerosis. Unlike apoE-deficient mice, LDL receptor–deficient mice and HuBTg mice do not develop lesions on a low-cholesterol, low-fat diet. The initial reliance on the very high cholesterol–cholic acid diet to produce lesions is problematic (as is true for the C57BL/6 model), as is the uncertainty about progression to the fibrous plaque lesion stage. It is encouraging that LDL receptor–deficient mice develop lesions on a Western-type diet, and perhaps with longer feeding periods these mice will develop the same type of fibrous plaque lesions seen in humans.

Applications of the Mouse Models

In principle, the mouse models of atherosclerosis (summarized in Table 1) can provide insights into lesion pathogenesis, genetic modifiers, and the influence of environment, hormones, and drugs on the disease. Although this work is largely in its infancy, the studies reported to date are promising. With regard to lesion pathogenesis, it is now possible to study the molecular events involved in monocyte attachment to endothelium, monocyte transmigration to the subendothelial space, subintimal foam cell formation, foam cell necrosis and the ensuing fibroproliferative reaction, and the roles of immune cells, cytokines, and their receptors in lesion progression. These studies can be carried out by documenting the molecules and cell types present in the lesions, crossbreeding the atherosclerotic mice with other mutant mice harboring specific defects in the same molecules or cells, and then noting suppression or enhancement of the atherosclerosis phenotype.

The foam cell lesions of both the apoE-deficient and LDL receptor–deficient mice contain oxidized epitopes of lipoprotein particles, accompanied by very high plasma levels of autoantibodies to oxidized lipoproteins (*10*, *13*). On the basis of in vitro studies and lesion immunohistochemistry, lipoprotein oxidation in the subendothelium has been hypothesized to be necessary for foam cell formation. These mouse models can be used to test this hypothesis in vivo. Support for the hypothesis has come from a recent pharmacological study in which an antioxidant, *N*,*N*′-diphenyl-1,4-phenylenediamine, was shown to decrease lesion area in apoE-deficient mice without affecting cholesterol levels (*14*). The mouse atherosclerosis models can also be used to identify molecules involved in lipoprotein oxidation through crossbreeding with other appropriate mutant mice. Similarly, experiments could be designed to test whether autoantibodies to oxidized lipoproteins participate in lesion pathogenesis or are a response to lesion development.

In another study, the role of the macrophage in lesion development was tested by crossbreeding apoE-deficient mice with mice that have the op mutation, a mutation in the gene that codes for macrophage colony stimulating factor (MCSF). MCSF influences monocyte and macrophage development, and mice with the op mutation have reduced levels of blood monocytes and tissue macrophages (*15*). Macrophages in the subendothelium have been hypothesized to protect against atherosclerosis by scavenging noxious materials such as oxidized lipoproteins, but they have also been hypothesized to contribute to foam cell formation and, by their death, to lesion progression. Lesions in mice that were doubly mutant (that is, apoE-deficient mice that also had the op mutation) were one-seventh the size of lesions in apoE-deficient mice, with almost no progression to the fibroproliferative stage, even though cholesterol levels in the doubly mutant mice were two to three times those in the apoE-deficient mice. These results strongly suggest that the net effect of the macrophage is proatherogenic. This hypothesis can be further tested by crossbreeding the apoE-deficient mice with mice that have mutations in other aspects of monocyte function.

The availability of mouse atherosclerosis models allows the use of a variety of techniques to identify genes that enhance or suppress the phenotype. In early studies, genes that might influence lipoprotein levels were expressed in transgenic mice on the C57BL/6 background. The mice were fed a very high cholesterol–cholic acid diet, and the effect of the transgene on the size of the lesions that formed at the aortic leaflets was assessed. In this model, expression of the human *apoA-I* gene, which codes for the major protein of HDL, led to raised HDL levels and reduced lesion area; coexpression of the human *apoA-I* gene and the human

Table 1. Summary of current mouse models of atherosclerosis.

Model	Atherogenic stimulus	Cholesterol level (diet)	Lesion type
C57BL/6	VLDL, LDL	200 to 300 (very high cholesterol, cholic acid)	Fatty streak (aortic leaflet)
ApoE deficiency	Chylomicron and VLDL remnants	400 to 600 (low cholesterol, low fat)	Fatty streak, progressing to fibrous plaque, at branch points of major vessels
		1500 to 2000 (Western type)	Same pattern as for low-cholesterol, low-fat diet, but with larger lesions and faster progression
ApoE Leiden	Chylomicron and VLDL remnants	1600 to 2400 (very high cholesterol, cholic acid)	Fatty streak, fibrous plaque
ApoE R142C	Chylomicron and VLDL remnants	370 (very high cholesterol, cholic acid)	Fatty streak
LDL receptor deficiency	IDL, LDL	1500 (very high cholesterol, cholic acid)	Fatty streak, progressing to necrotic core but without fibrous cap
		1200 (Western type)	Fatty streak
HuBTg	LDL	300 to 500 (very high cholesterol, cholic acid)	Fatty streak, progressing to necrotic core but without fibrous cap

apoA-II gene, which codes for the second most abundant HDL protein, was less protective (*16*). These results suggest that both the amount and the apolipoprotein content of HDL influence susceptibility to atherosclerosis. In another study, outbred transgenic mice expressing mouse *apoA-II* developed lesions when they were fed the very high cholesterol–cholic acid diet, whereas control mice did not; this result again suggested that overexpression of *apoA-II* is proatherogenic (*17*). More recently, mice expressing the human *apoA-I* transgene were bred with apoE-deficient mice, and the offspring were fed a low-cholesterol, low-fat diet; at 4 months of age, fatty streak lesions were almost totally suppressed, whereas at 8 months some small fatty streaks had appeared but fibrous plaques were suppressed (*18*). Thus, *apoA-I* expression led to decreased lesion area and inhibited lesion progression. These same mice showed a strong inverse correlation between HDL cholesterol levels and lesion size. This effect of HDL was independent of the effect of non-HDL cholesterol levels on lesion size.

The relation between low HDL cholesterol levels and increased atherosclerosis observed in human epidemiological studies has been attributed largely to the association of low HDL cholesterol levels with high levels of atherogenic apoB-containing lipoproteins, such as VLDL, IDL, small dense LDL, and postprandial particles. These mouse studies suggest that HDL has an additional independent protective effect, perhaps by accelerating reverse cholesterol transport or directly protecting the vessel wall against noxious atherogenic stimuli. Evidence for one or both of these proposed mechanisms of HDL action can now be sought in the mouse atherosclerosis models.

Another potential atherosclerosis modifier is the cholesteryl ester transfer protein (*CETP*) gene. This gene codes for a plasma protein that mediates the exchange of triglycerides in VLDL for cholesterol esters in HDL. HDL cholesterol levels have been inversely correlated with plasma CETP activity in a variety of clinical studies. Although mice (unlike humans) do not have plasma CETP activity, transgenic mouse lines have been created with either the human or monkey *CETP* genes, and the effects of this gene on atherosclerosis in mouse models have been evaluated. Expression of the monkey *CETP* gene in C57BL/6 mice that were fed the very high cholesterol–cholic acid diet led to reduced HDL cholesterol levels, raised non-HDL cholesterol levels, and increased lesion size (*19*). However, expression of the human *CETP* gene in transgenic mice that were made hypertriglyceridemic by expression of the human *apoC-III* gene resulted in reduced lesion size (*20*). These experiments suggest that CETP can be proatherogenic or antiatherogenic, depending on other factors that determine the lipoprotein profile.

Another candidate gene that has been assessed for its role in atherogenesis is *apo(a)*. Apo(a) is a large glycoprotein that forms a disulfide bond with the apoB moiety of LDL; the resulting particle is called Lp(a). Clinical studies have yielded conflicting data concerning the role of Lp(a) levels in coronary heart disease susceptibility. When outbred transgenic mice expressing *apo(a)* were fed the very high cholesterol–cholic acid diet, they developed small fatty streaks in the region of the aortic valve leaflets; these lesions were not present in control mice (*21*). Because human apo(a) does not bind to mouse apoB and because the apo(a) in these mice was not lipoprotein-associated, these results were interpreted to mean that apo(a) is directly atherogenic. More recently, *apo(a)* transgenic mice were crossbred with HuBTg mice (*12*, *22*). Lp(a) does form in these mice, and when they were fed the very high cholesterol–cholic acid diet, they developed lesions that were twice the size of those in the HuBTg mice. These studies indicate that apo(a) and Lp(a) are marginally atherogenic. However, further studies with transgenic mice expressing higher levels and different isoforms of apo(a) are needed, as are tests of atherogenicity in the context of more natural diets.

Induced mutant mouse models have been used to test the atherogenicity of apoE itself. For example, mice with only one inactivated allele of apoE developed atherosclerosis when they were fed the very high cholesterol– cholic acid diet; this finding suggests that even at half its normal production, apoE is atherogenic in the face of a severe dietary challenge (*23*). In other studies, bone marrow transplantation into apoE-deficient mice has been shown to correct the hyperlipidemia and prevent atherosclerosis, which indicates that reconstitution of macrophage apoE is sufficient to correct the metabolic defect (*24*). Finally, local production of apoE by expression of a transgene in macrophages or in the blood vessel wall was found to diminish atherosclerosis independent of its cholesterol-lowering effect (*25*). Thus, local production of apoE may play a special role in the prevention of atherosclerosis.

The mouse atherosclerosis models can also be used to test the effects of environment, hormones, and drugs on atherogenesis. In the apoE-deficient and LDL receptor–deficient mice, lesion size increases as the diet is changed from low cholesterol–low fat to high cholesterol–high fat (*6*, *10*). Popular, but as yet unproved, theories about the effects of various macro- and micronutrients on atherogenesis can also be tested. With regard to hormonal effects on atherosclerosis, among the LDL receptor–deficient mice on the very high cholesterol–cholic acid diet, the lesion area throughout the vascular tree is apparently greater in males than in females; this model may prove useful in studying the mechanisms by which sex hormones affect atherogenesis (*26*). Finally, these mouse models can be used to test for drugs that inhibit atherogenesis. The availability of large numbers of atherosclerosis-prone mice will allow relatively inexpensive and thorough preclinical testing of new candidate drugs.

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RESEARCH ARTICLE

Inhibition of atherosclerosis in apolipoprotein-E-deficient mice following muscle transduction with adeno-associated virus vectors encoding human apolipoprotein-E

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Apolipoprotein E (apoE) is a multifunctional plasma glycoprotein involved in lipoprotein metabolism and a range of cell signalling phenomena. ApoE-deficient (apoE$^{-/-}$) mice exhibit severe hypercholesterolaemia and are an excellent model of human atherosclerosis. ApoE somatic gene transfer and bone marrow transplantation in apoE$^{-/-}$ mice results in reversal of hypercholesterolaemia, inhibition of atherogenesis and regression of atherosclerotic plaque density. Replication defective adeno-associated virus vectors (rAAVs) are an attractive system currently in clinical trial for muscle-based heterologous gene therapy to express secreted recombinant plasma proteins. Here we have applied rAAV transduction of skeletal muscle to express wild-type (ε3) and a defective receptor-binding mutant (ε2) human apoE transgene in apoE$^{-/-}$ mice. In treated animals, apoE mRNA was present in transduced muscles and, although plasma levels of recombinant apoE fell below the detection levels of our ELISA (ie <10 ng/ml), circulating antibodies to human apoE and rAAV were induced. Up to 3 months after a single administration of rAAV/apoE3, a significant reduction in atherosclerotic plaque density in aortas of treated animals was observed (approximately 30%), indicating that low-level rAAV-mediated apoE3 expression from skeletal muscle can retard atherosclerotic progression in this well-defined genetic model.

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Keywords: *gene therapy; apolipoprotein E; atherosclerosis; AAV*

Introduction

Atherosclerosis is the primary cause of death in industrialised countries and is rapidly becoming the leading cause of death and disability in the rest of the world.[1,2] In the United Kingdom, 38% of premature deaths in men and 30% of premature deaths in women are due to cardiovascular disease.[3] Atherosclerosis is caused by the inflammatory deposition of lipoprotein-derived cholesterol into the artery wall resulting in the development of fatty streaks that go on to form atherosclerotic plaques, leading to local occlusion of the arteries and consequently cardiovascular disease.[4] A major component within lipoprotein metabolic pathways that plays a role in preventing development of atherosclerotic lesions is apolipoprotein E (apoE). ApoE is a major glycoprotein constituent of lipoprotein particles involved in the transport of excess cholesterol from the peripheral tissues to the liver for excretion, a process known as reverse cholesterol transport.[5–7] ApoE is the ligand for the low-density lipoprotein receptor (LDL-R) and the LDL-R-related protein (LRP) expressed by the liver. Interaction of apoE with cell-surface heparan sulphate proteoglycans (HSPG) is also the first stage in clearance of remnant particles by the liver and is part of the LRP lipoprotein clearance pathway.[8] Through these interactions, apoE mediates the hepatic clearance of cholesterol-rich dietary chylomicron remnants, very low-density lipoproteins (VLDLs), intermediate density lipoproteins (IDLs) and a subclass of high-density lipoproteins (HDLs) from the circulation.[6,9] Also apoE plays a part in cholesterol efflux from cells in atherosclerotic lesions via γLpE, an immature HDL particle in which apoE is the sole protein component.[10–12]

There are three major variant isoforms of apoE, designated apoE2, apoE3 and apoE4 (encoded by polymorphic alleles, ε2, ε3 and ε4), of which apoE3 is the most common functional isoform.[6,13] Genetic deficiency in apoE and also the presence of apoe2 and apoe4 genes in the population are associated with predisposition to atherosclerosis and Alzheimer's disease, respectively.[13,14] In particular homozygosity for apoe2 is associated with type III hyperlipoproteinaemia, which is characterised by elevated levels of the chylomicron remnants, VLDL and IDL, with concomitant increases in cholesterol and triglyceride levels and with consequent predisposition to premature atherosclerosis.[15]

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The apoE$^{-/-}$ transgenic mouse has been used extensively as an animal model for atherosclerosis as it develops severe hypercholesterolaemia and atherosclerotic lesions similar to those found in humans.[16,17] ApoE is normally expressed predominantly by hepatocytes and macrophage cells of haematopoietic lineage, and transplantation of normal bone marrow cells into apoE$^{-/-}$ mice results in increased lipoprotein clearance, reversal of hypercholesterolaemia, and protection against development of atherosclerosis.[18,19] Liver-directed somatic gene transfer with recombinant adenovirus vectors (rAds) containing the apoE3 cDNA has also been shown to result in transient normalisation of lipoprotein and cholesterol plasma profiles and inhibition of atherosclerotic lesion development.[20,21] ApoE2 is less efficient at correcting the hyperlipidaemic phenotype compared with apoE3, due to reduced efficiency in mediating lipoprotein plasma clearance and liver uptake, but nevertheless has been shown to exhibit residual anti-atherogenic activity and retardation of atherosclerotic lesion development in apoE$^{-/-}$ mice, following liver- and muscle-directed gene transfer.[22,23]

A major limitation of studies utilising rAd vectors to mediate apoE gene transfer has been the induction of adverse host immune and inflammatory responses,[24,25] which lead to only transient transduction and to rapid target cell elimination. For studies showing quantitative regression of atherosclerotic plaques, recourse to immunodeficient athymic apoE$^{-/-}$ mice was required.[26] Recombinant adeno-associated viruses (rAAVs) are attractive candidate vectors for gene therapy as they are defective and non-pathogenic,[27] can transduce both dividing and non-dividing cells, and are devoid of viral structural genes which diminishes the risk of adverse host immune responses.[28,29] In particular, the use of skeletal muscle as a heterologous site for gene delivery with rAAV vectors has gained increasing importance as a platform for the secretion of therapeutic transgene products into the circulation. Thus, skeletal muscle is well vascularised, accessible and actively secretory.[30] Several studies have shown that after intramuscular injection of rAAV vectors, there was a gradual increase in transgene expression over several weeks that reached a plateau of sustained expression.[29,31–36] In a study by Xiao *et al*[31] *LacZ* expression was maintained for more than 1.5 years with no evidence of immune elimination of transduced myofibres or response against the reporter transgene product.

In the present study we have constructed and tested rAAV-based vectors containing human apoE transgenes for heterologous muscle-based expression in apoE$^{-/-}$ mice. ApoE mRNA was present in transduced muscles, and despite low plasma levels of recombinant products, circulating antibodies to human apoE and rAAV were induced. In the case of rAAV encoding human apoE3, but not apoE2, a single intramuscular administration resulted in significant (approximately 30%) reduction in atherosclerotic plaque density in the aorta of treated animals. The results indicate that heterologous, muscle-based and rAAV-mediated expression of human apoE3 is protective against atherosclerotic progression in this well-defined genetic model, even in the presence of a humoral immune response directed against the transgene product and virus capsid proteins.

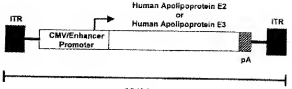

Figure 1 Schematic representation of rAAV vectors for expression of human apoE2 and apoE3. The rAAV vectors were engineered with an expression cassette containing the CMV immediate–early promoter/enhancer and SV40 intron (splice donor/splice acceptor), driving expression of either apoE2 (rAAV/apoE2) or apoE3 (rAAV/apoE3) cDNAs, followed by an SV40 polyadenylation signal (pA). ITR, inverted terminal repeat; SD/SA, SV40 intron.

Results

Construction and in vitro testing of rAAV vectors expressing human apoE transgenes

Recombinant rAAV vectors containing a transgene expression cassette consisting of the full cytomegalovirus (CMV) immediate–early enhancer/promoter driving expression of the human apo-e2 or apo-e3 cDNAs were used to assess the ability of the rAAV vectors to infect and engineer cells to express the transgenes (Figure 1). HeLa and 293-T cells were infected with rAAV/apoE2 (Figure 2) or rAAV/apoE3 (data not shown) at high MOIs and 3 days later culture supernatants were harvested. Western blot analysis demonstrated that human apoE had accumulated in the culture supernatants even in the absence of the rAd helper virus Ad-βGal (Figure 2).

In vivo transduction of skeletal muscle with rAAV/apoE2 and rAAV/apoE3 vectors in apoE$^{-/-}$ mice

ApoE$^{-/-}$ mice at 6–8 weeks of age were injected with 2.5 × 10^{10} virus particles of either rAAV/apoE2 or rAAV/apoE3 vectors into both tibialis anterior (TA) muscles. Tail vein bleeds were taken at 2, 4, 6, 10 and 14 weeks, and plasma samples evaluated for the presence of either human apoE2 or apoE3 proteins by ELISA and Western blot analysis. At all time-points the levels of transgene products in the circulation of the treated animals were undetectable and thus fell below the sensi-

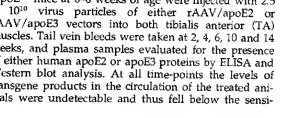

Figure 2 Secretion of recombinant apoE (34 kDa) from rAAV-transduced HeLa and 293-T cells. HeLa and 293-T cells were infected with rAAV/apoE2 at MOIs of 100 000 and 30 400, respectively. The HeLa cells were coinfected with a helper adenovirus (Ad-βGal) at an MOI of 50 where indicated. Western blotting analysis was performed upon culture supernatants 3 days after infection. Similar results were obtained with the rAAV/apoE3 vector (data not shown).

tivity limits of these methods (ie <10 ng/ml). Muscle tissues from the site of rAAV injection were collected at 14 weeks and total RNA extracted. RT-PCR analysis of muscle RNAs, using human apoE-specific primers common to both the ε2 and ε3 isotypes, demonstrated the presence of an appropriate 227-bp amplification product in all treated samples, but not in control muscles (Figure 3a). In addition, restriction fragment length polymorphism isotyping confirmed that the RT-PCR products were derived from the respective apoE transcripts, as *Hha*I digestion produced restriction fragments of the expected size for the apoE2 and apoE3 genotypes (Figure 3b).

Humoral immune response against human apoE transgene products and rAAV virions

Plasma samples from groups of rAAV-treated mice at the various time-points, were pooled and screened for antibodies against transgene product and rAAV capsid proteins by Western blot analysis against partially purified recombinant human apoE and rAAV vector, respectively. ApoE antibodies were detected from 4 to 6 weeks after rAAV administration and levels increased throughout the course of the experiment (Figure 4a). For the detection of anti-AAV antibodies plasmas were screened against Western blots of intact rAAV/apoE virus stock. Here AAV capsid antibodies were clearly present again, from 2 weeks after injection, increasing marginally up to 10 to 14 weeks when the animals were killed (Figure 4b). Antibodies to human apoE or AAV capsid proteins were not detected in plasma samples from the mock-injected control animals.

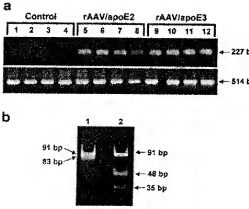

Figure 3 Characterisation of human apoE2 and apoE3 gene expression in rAAV transduced tibialis anterior (TA) muscles of apoE$^{-/-}$ mice. Animals were given intramuscular injections of either rAAV/apoE2, rAAV/apoE3 or were mock injected and 14 weeks later total RNA from treated TA muscles were isolated and DNase treated. (a) RT-PCR amplification of a 227 bp product utilising a primer set that is common to both apoE isoforms. To determine the quality and loading of the total RNA preparations a mouse β-actin primer set was used that generated a 514 bp product. (b) ApoE isoform genotyping of RT-PCR products by HhaI digestion followed by 10% PAGE. The predicted fragments for apoE2 are 83 bp and 91 bp (lane 1), and for apoE3 expected fragment sizes are 35 bp, 48 bp and 91 bp (lane 2). Representative HhaI digested RT-PCR products derived from rAAV/apoE2 and rAAV/apoE3 injected TA muscle are shown.

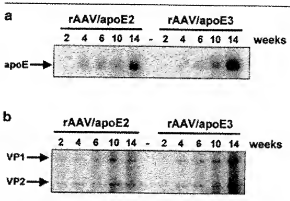

Figure 4 Generation of a humoral immune response to apoE transgene products and AAV capsid proteins in transduced apoE$^{-/-}$ mice. Western blots of partially purified human apoE (a) and rAAV protein preparations (b) were used to screen pooled plasmas from each bleed of rAAV/apoE2 and rAAV/apoE3-treated animals. The arrows indicate the expected sizes of human apoE (34 kDa) and the AAV capsid proteins VP1 (87 kDa) and VP2 (72 kDa).

Plasma cholesterol levels in mice transduced with rAAV/apoE vectors

Plasma samples from individual mice were evaluated for total cholesterol levels at 2, 4, 6, 10 and 14 weeks after intramuscular injection of rAAV/apoE vectors. At 2 weeks, plasma samples from animals treated with rAAV/apoE2 or rAAV/apoE3 demonstrated mean total cholesterol levels of 346 mg/dl and 435 mg/dl, respectively, with the control group giving a value of 440 mg/dl (Figure 5a; the normal range for wild-type mice is 80–150 mg/dl[37,38]). Statistical analysis established that the reduction in plasma cholesterol in the rAAV/apoE2 treated group was significant at the 2-week time-point only (P = 0.003). This initial disparity between the rAAV/apoE2-treated and the control group was only transient and by 6 weeks the difference in cholesterol levels between these two groups was insignificant. No significant effect of rAAV/apoE3 transduction on plasma cholesterol levels was observed. Upon analysis of the lipoprotein particle distribution at 2 and 10 weeks after rAAV/apoE vector administrations, no appreciable changes were observed (Figure 5b).

Analysis of atherosclerotic lesion density in aortas of rAAV/apoE transduced mice

To investigate the effect of human apoE gene transfer upon atherosclerotic lesion development, apoE$^{-/-}$ mice were injected with rAAV vectors and killed at 14 weeks. Aortas were dissected, opened along their entire length, fixed intact and treated with Oil-Red-O which specifically stains lipid-laden atherosclerotic lesions (Figure 6a). Those mice treated with the rAAV/apoE3 vector demonstrated reduced levels of Oil-Red-O stained atherosclerotic and fatty streak lesions compared with the control group (Figure 6a and b). Aortas of rAAV/apoE3-treated animals had a total lesion coverage area of 16.4 ± 1.6%, and the control group gave a mean value of 23.0 ± 2.2% (mean ± s.e.m.; Figure 6b). Therefore the rAAV/apoE3-treated group of animals demonstrated a significant reduction in aortic lesion density of 29% (P = 0.033) compared with control apoE$^{-/-}$ mice. In the case of animals injected with rAAV/apoE2, the mean aortic lesion area

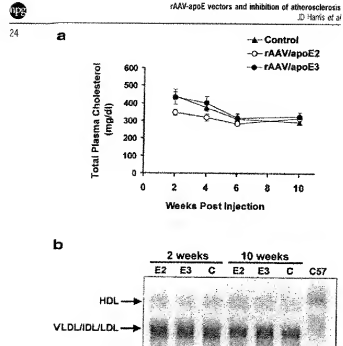

Figure 5 Evaluation of total plasma cholesterol levels and lipoprotein distribution in apoE$^{-/-}$ mice after intramuscular injection of rAAV/apoE vectors. (a) Total plasma cholesterol levels were determined for individual animals at 2, 4, 6 and 10 weeks after injection. A statistically significant lowering of cholesterol was observed at 2 weeks in the rAAV/apoE2-treated animals ($P = 0.005$). C57Bl/10 (normal) mice fed on the same chow diet as the animals in this study routinely gave total plasma cholesterol levels of 90 to 100 mg/dl (data not shown). (b) Pooled plasma samples (2 μl) taken 2 and 10 weeks after intramuscular injection of the rAAV/apoE vectors, were subjected to 0.8% agarose gel electrophoresis and the separated lipoproteins stained with Sudan black. C, plasma (2 μl) from pooled plasmas of the untreated apoE$^{-/-}$ mice. C57, plasma (2 μl) from a C57Bl/10 mouse.

was also reduced (20.8 ± 1.28%), but this was not significant compared with the control group ($P = 0.39$).

Discussion

ApoE$^{-/-}$ mice are characterised by having severe hypercholesterolaemia with plasma cholesterol levels of between 400 to 800 mg/dl, developing premature atherosclerosis on a normal chow diet, and are a relevant model of human atherosclerotic disease.[17,37,38] The restoration of normal cholesterolaemic levels and protection against atherosclerosis has been achieved in apoE$^{-/-}$ animal models by the gene transfer of human apoE, using retroviral, adenoviral and plasmid expression vectors.[22,25,26,39–41] As a platform for secretion of therapeutic proteins into the circulation, muscle has been used extensively in rAAV-mediated gene transfer yielding sustained transgene expression in the absence of immune clearance of transduced cells.[29,31–35]

We have examined the feasibility of targeting muscle as a platform for the secretion of anti-atherogenic proteins by the transfection of mouse myoblast cultures with rAAV-based plasmid vectors containing apoA1 and LCAT cDNAs.[17] A significant improvement in secretion of apoA1 and LCAT and retention of vector sequences by transfected cells was observed using the rAAV-based vectors compared with conventional plasmid expression vectors. We also demonstrated that cultured mouse C2C12 cells transfected with a plasmid expression vector containing the CMV promoter driving expression of the human apoE2 or apoE3 cDNAs, can express and secrete human apoE.[23] The secreted proteins became incorporated into spherical lipoprotein particles similar to those involved in cholesterol efflux,[10–12] thereby providing evidence for the functional activity of the expressed proteins. In addition, intramuscular injection of an apoE expression plasmid in apoE$^{-/-}$ mice, resulted in secretion of apoE and retardation of aortic atherosclerotic lesions.[23] In support of these findings, Rinaldi *et al*[43] also performed intramuscular injections of a plasmid expressing the apoE cDNA, resulting in reduction of serum cholesterol, improved hepatic clearance of VLDL, IDL and LDL cholesterol and an increase in HDL cholesterol.

Our present studies aim to combine the efficient rAAV-mediated transduction of muscle with the anti-atherogenic properties of apoE. Intramuscular injection of apoE$^{-/-}$ mice with the rAAV/apoE vectors resulted in apoE expression below ELISA and Western blot detection limits. However, indirect evidence for apoE expression was demonstrated by the generation of circulating apoE antibodies and detection of apoE transgene mRNAs in muscle tissues 4–6 weeks and 14 weeks, respectively, after transduction. Previously, we have shown that following intramuscular injection of plasmids containing the apoE transgene cassettes employed in this study, apoE2 and apoE3 transgene products were effectively secreted into the circulation.[23] Furthermore, apoE$^{-/-}$ mice subjected to muscle transduction with rAAV/apoE3 vectors exhibited significant reduction in aortic atherosclerotic lesion density even in the absence of altered plasma cholesterol levels. Herzog *et al*[34,43] have previously detected high-titre antibodies to human factor IX (hFIX) after muscle transduction with rAAV/hFIX vectors in mice and dogs, but other studies have demonstrated a lack of an immune response against rAAV transgene products.[31–33,44] In one instance, intramuscular injection of an rAAV vector expressing β-galactosidase resulted in an immune response after 4 weeks, followed by immune tolerance and long-term expression of the foreign protein.[35] According to Joos *et al*,[44] rAAV-mediated intramuscular gene transfer evades the immune system by the inability of the vector to transduce antigen-presenting cells. Thus it is likely that only when the transgene product is secreted or membrane-associated may a humoral immune response develop, and the reported variability in the immune responses may reflect differences in immunogenicity of foreign proteins and the immunology of animal model/strains. In a recent study, apoE$^{-/-}$ mice were treated with a first generation adenovirus containing human apoE cDNA and complete regression of atherosclerosis was demonstrated 6 months after injection.[76] As these animals were immune deficient, no immune response was mounted against the adenovirus vector and recognition of human apoE as a neoantigen was minimal which resulted in prolonged transgene expression.

Previous studies have demonstrated that low levels of apoE in apoE$^{-/-}$ mice can inhibit atherosclerosis without reducing cholesterol levels. Thorngate *et al*[45] generated

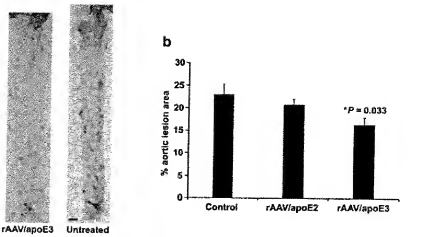

*Figure 6 Reduction in aortic atherosclerotic lesions in apoE$^{-/-}$ mice treated with rAAV/apoE2 and rAAV/apoE3. Animals were killed 14 weeks after rAAV vector administration and the aortas removed, pinned out en face on to cork bess and stained with Oil-Red-O. (a) Representative aortas from the rAAV/apoE3 treated and untreated animals. The red-stained areas denote the accumulation of neutral lipids in the vessel wall. Scale bar = 1 mm. (b) Mean percent aortic lesion areas of the untreated and rAAV treated animals. *Comparison of the mean of the rAAV/apoE3-treated group against the mean of the control group using a two-tailed unpaired t test.*

transgenic apoE$^{-/-}$ mice expressing apoE (approximately 1 μg/ml plasma: <2% of wild-type levels) from the adrenal glands, and although hypercholesterolaemia was not corrected, significant protection against atherosclerosis occurred. Inhibition of atherosclerotic lesion development has also been achieved by retrovirus-mediated *ex vivo* transduction and re-implantation of apoE$^{-/-}$ bone marrow stem cells yielding plasma apoE3 levels of approximately 1% of normal without correction of hypercholesterolaemia.[39] In this case, reduction in atherosclerosis was reported only in young mice (5–10 weeks) and not in older animals (13 to 26 weeks). Our results support this finding as the mice evaluated in the present study were treated at 6–8 weeks of age and therefore, low plasma apoE levels appeared to be effective at inhibiting lesion development in the early stages of atherogenesis in the absence of lowering plasma cholesterol. In this respect, local effects of apoE *in situ*, at the atherosclerotic lesion site may be an important parameter in controlling atherosclerotic lesion progression, secondary to the lowering of plasma cholesterol levels. Notably, deposition of recombinant apoE3 within atherosclerotic lesions has been demonstrated following rAd-mediated liver transduction,[22] and associated with macrophages following bone marrow transplantation.[39]

A defining stage in the development of atherosclerotic lesions that precedes the attachment of monocytes to the vessel wall, is the accumulation of remnant lipoproteins on the intimal extracellular matrix which occurs as early as 3 weeks of age in apoE$^{-/-}$ mice.[46] The presence of low levels of apoE in the circulation may prevent the recruitment of the remnant lipoproteins to the vessel wall and therefore protect against the development of atherosclerosis.[45] This may explain how low levels of human apoE in the circulation of apoE$^{-/-}$ mice can protect against early lesion development. Other anti-atherogenic properties of apoE include the inhibition of platelet aggregation,[47] lymphocyte activation and proliferation,[48] inhibition of smooth muscle cell proliferation,[49] suppression of vascular cell adhesion molecule-1 (VCAM-1) that is involved in the recruitment of monocytes to the site of lesion development,[50] and anti-oxidant activity that prevents the formation of oxidised LDL, which is a major factor in foam cell formation.[14,51] The significant reduction in lesion development of animals treated with rAAV/apoE3 compared with those treated with rAAV/apoE2, may reflect the association of apoE2 with type III hyperlipoproteinaemia, where individuals homozygous for apoE2 are prone to developing premature atherosclerosis. ApoE plays a major part in cholesterol efflux from cells in atherosclerotic lesions via γLpE, an immature HDL particle of which apoE is the sole protein component.[10–12] The efficiency of this process is isoform-dependent with apoE3 being more efficient in forming γLpE particles, reflected by the much enhanced ability of ε3/ε3 plasma samples to mobilise cell-derived cholesterol compared with ε2/ε2 subjects.[13] A direct comparison of apoE2, apoE3 and apoE4 isoforms by adenoviral gene transfer into apoE$^{-/-}$ mice, demonstrated the impaired ability of apoE2 to clear VLDL, IDL and remnant lipoproteins from the circulation compared with apoE3 and apoE4,[22,40] due to reduced affinity for the LDL-R and LRP.[6,52,53]

In addition to a humoral immune response against apoE2 and apoE3, antibodies directed against the virus capsid proteins VP1 and VP2 were present 4–6 weeks after vector administration. Over the course of the analysis, anti-AAV antibody titres rose marginally, compared with a more pronounced increase of antibodies against apoE. This may reflect the ongoing expression of apoE2 and apoE3 transgene products from muscle sites, compared with only the exogenous source of virus antigen. While the presence of neutralising antibodies to AAV would likely preclude efficient readministration of rAAV vectors, transient immuno-blockade at the time of rAAV vector administration can result in successful rAAV vector readministration.[54]

This study has demonstrated that expression of low levels of human apoE3 in apoE$^{-/-}$ mice by intramuscular injection of rAAV vectors leads to a significant reduction in atherosclerotic aortic lesion development, and confirms previous studies.[23,39,41,48] The low circulating levels of apoE in the present study indicate that significant improvements are necessary in order to achieve complete protection against atherosclerosis using muscle-based rAAV-mediated gene transfer. In this respect, the current rAAV vectors are based upon AAV serotype-2, and recent studies indicate muscle expression following rAAV transduction can be increased by up to 100-fold using vectors based upon AAV serotypes 1 and 5.[58] In addition, targeting slow-twitch muscle fibres, as opposed to fast-twitch muscle fibres (which predominate in the TA muscle sites used here) may also improve rAAV-mediated transduction due to increased levels of cell surface HSPG, a primary receptor for AAV.[56]

Materials and methods

rAAV vector construction

The rAAV plasmid vectors were constructed using pNTC3-CMVβ,[23,42] which contains the 5'- and 3'- ITRs between which is a CMV promoter/LacZ expression cassette. The *LacZ* gene was deleted from pNTC3-CMVβ by *NotI* and *EcoRV* digestion, and replaced with the human apoE2 and apoE3 cDNAs excised from their respective pUC-based vectors,[57] using *HincII*/*SmaI* digestion then blunt-ended, producing the rAAV plasmid vectors pAAV/apoE2 and pAAV/apoE3.

rAAV production and purification

For the production of rAAV vectors, pAAV/apoE2 or pAAV/apoE3 were co-transfected with the helper plasmid pDG into 293-T cells,[58,59] at a molar ratio of 1:1. The pDG plasmid contains the rep and cap genes and adenovirus helper functions required for rAAV production. Cell lysates were prepared 2 days after the transfection and rAAV particles purified by iodixanol stepped gradient centrifugation as previously described.[60] Final virus stocks were stored in PBS-MK (phosphate buffered saline, 5 mM $MgCl_2$, 12.5 mM KCl) and were titred by determining the rAAV vector genome copy number using DNA dot-blot hybridization.[61] From 10 15-cm dishes of transfected 293-T cells, between 5×10^{11} and 2×10^{12} rAAV particles were isolated and purified on a routine basis.

Culture and rAAV/apoE infection of HeLa and 293-T cells

HeLa and 293-T cells were routinely passaged to subconfluency in DMEM containing 10% fetal bovine serum (Life Technologies, Paisley, UK), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B at 37°C and 8% CO_2. Hela and 293-T cells were seeded in six-well plates at 3×10^5 and 8×10^5 cells per well, respectively. The following day HeLa and 293-T cells were infected with the rAAV vectors at an MOI of 100 000 and 30 400, respectively. Cell culture supernatants were replaced with 700 μl of infection mix containing 3.8×10^{10} rAAV/apoE2 virus particles in PBS/$MgCl_2$/$CaCl_2$ (phosphate buffered saline containing 49 mM $MgCl_2$ and 68 mM $CaCl_2$). As indicated, a first generation adenovirus containing the *LacZ* gene (Ad-βGal) was used as helper adenovirus at an MOI of 50. After 3 h at 37°C/8% CO_2 the infection mixes were replaced with fresh culture medium and the culture media was harvested 3 days later for analysis of secreted human apoE proteins.

rAAV vector administration in vivo

Female apoE$^{-/-}$ mice provided by GlaxoSmithkline (Stevenage, UK), were generated by inactivation of the mouse apoE locus through homologous recombination as previously described.[16] The animals were maintained on a normal chow diet and blood samples were taken after a 4 h fast from the tail vein. The rAAV vector stocks were diluted to 6.25×10^{11} viral particles/ml using PBS-MK and 40 μl (2.5×10^{10} virus particles) of either rAAV/apoE2 or rAAV/apoE3 were injected into both TA muscles of mice anaesthetised by intra-peritoneal administration of a 25% solution of hypnorm and hypnovel (3 μl per gram body weight). Control animals were injected with PBS-MK only. Tail vein bleeds were carried out where approximately 50 μl of blood was anti-coagulated with sodium citrate. Plasma samples were stored at −80°C.

Detection of human apoE in culture supernatants and plasma from treated apoE$^{-/-}$ mice by Western blotting analysis

Culture supernatant from transduced cells or plasma samples, were denatured by the addition of SDS-PAGE sample buffer containing 2.5% (v/v) β-mercapto-ethanol and heating at 100°C for 5 min. Samples were then subjected to 4–12% SDS-NuPAGE gradient electrophoresis (Invitrogen/Novex, Groningen, The Netherlands), and resolved proteins transferred to an ECL-nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). Nitrocellulose blots were incubated with goat anti-human apoE antibody (1:3000 dilution; Biogenesis Ltd, Poole, UK) followed by an anti-goat-HRP secondary antibody (1:3000 dilution; Sigma, Poole, UK). Blots were developed using the ECL detection system (Amersham Pharmacia Biotech).

Enzyme-linked immunosorbent assay (ELISA) for quantification of human apoE

Human apoE2 or apoE3 in mouse plasma was detected by a two-antibody sandwich ELISA. Polyclonal goat anti-human apoE antibodies were used both for capture (DiaSorin Inc, Stillwater, MN, USA) and after biotinylation using a commercial kit (Amersham Pharmacia Biotech), for detection (Biogenesis Ltd, Poole, Dorset). Purified human apoE (Technoclone Ltd, Dorking, Surrey) was used as a standard. Standard and mouse plasma samples were diluted in assay buffer, 150 mM NaCl, pH 7.4 containing 0.5 % (w/v) bovine serum albumin, 0.05 % (w/v) gamma-globulin, 0.01 % (v/v) Tween-40 and 50 mM Tris; the detection limit was 10 ng/ml.

RT-PCR analysis

Total RNA was isolated from TA muscle that had been frozen in liquid nitrogen then stored at −80°C immediately after removal from the animals. Each muscle was thawed out in 0.5 ml of RNAlater (Ambion, Abingdon, UK) and sliced into small pieces, followed by isolation of total RNA with Trizol Reagent (Life Technologies),

according to the manufacturer's instructions. DNase treated RNA (1 μg) was reverse transcribed with Superscript II RNase H- Reverse Transcriptase (Life Technologies) and an oligo $(dT)_{12-18}$ primer at 42°C, following the manufacturer's instructions. The derived cDNA (1 μl from a 20 μl reaction) was used to amplify a 227 bp product using a primer set specific for the human apoE sequence. Each reaction contained 25 pmol of each primer (sense: 5'-TCCAAGGAGCTGCAGGCGG CGCA-3'; antisense: 5'-ACAGAATTCGCCCCGGCCTGG TACACTGCCA-3'), 200 μM of each deoxynucleotide triphosphate, 10% DMSO, 0.5 μl Pfu Turbo polymerase and 10× reaction buffer for Pfu Turbo polymerase (Stratagene, Amsterdam, The Netherlands) in a final volume of 50 μl. The reaction conditions were 94°C for 5 min followed by the addition of the polymerase; then 30 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 1 min followed by a final extension time of 72°C for 10 min. To confirm the quality of RNA preparations, a mouse β-actin primer set (Stratagene) was used to amplify a 514 bp product from 2 μl of cDNA. Each reaction contained 50 pmol of each primer, 200 μM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$ and 10× reaction buffer (Life Technologies) for Taq DNA polymerase. The reaction conditions were 94°C for 45 s followed by the addition of the polymerase; then thermal cycling was carried out for 30 cycles (45 s 94°C; 45 s 60°C; 90 s 72°C), followed by a final extension time of 72°C for 10 min. The PCR products were visualised by electrophoresis on a 1.8% agarose gel. Restriction enzyme apoE isoform genotyping was performed to demonstrate that the amplified products were specifically derived from rAAV/apoE2 and rAAV/apoE3.[62] Briefly, gel-purified PCR products were subjected to digestion with the restriction enzyme *Hha*I (New England Biolabs, Hitchin, UK) followed by 20% PAGE and then stained with ethidium bromide to visualise the restriction fragments.

Detection of anti-ApoE and anti-AAV antibodies by Western blotting analysis

For the detection of anti-human apoE antibodies in mouse plasma, human apoE3 was partially purified from CHO cells stably expressing the protein (CHO-apoE3).[63] CHO-apoE3 cells were cultured in CD-CHO medium (Life Technologies), which requires no addition of serum and contains 20 times less protein than conventional growth media. Human apoE3 was partially purified from the culture supernatant by concentration using a Vivaspin 30 kDa filter (Vivascience Ltd, Binbrook, UK), followed by dialysis against PBS. Human apoE3 protein (1 μg) was denatured by the addition of SDS-PAGE sample buffer containing 2.5% (v/v) β-mercaptoethanol and heating to 100°C for 5 min. Then the protein was loaded into a preparative well of a 4–12% SDS-NuPAGE (Invitrogen/Novex) and subjected to electrophoresis. For the detection of antibodies against the virus vector, 2.25 × 10^{11} viral particles of rAAV/apoE3 was used in place of the partially purified human apoE3. The electrophoresed proteins were transferred to a Hybond-PVDF membrane (Amersham Pharmacia Biotech) and placed in a Mini-PROTEAN II multiscreen apparatus (BioRad, Hemel Hempstead, UK). Equal volumes of mouse plasma from each animal within each treatment group were pooled and 2.5 μl was diluted in 600 μl of incubation buffer (TBST containing 2.5% milk powder, 0.05% Tween-20 and 0.2% 2-chloroacetamide) and this was used to screen for antibodies against human apoE. The same primary incubation buffer was used for the detection of anti-AAV capsid proteins. As positive controls for the detection of anti-apoE and anti-AAV antibodies, a mouse monoclonal anti-human apoE3 antibody,[64] and an anti-AAV antibody that recognises VP1, VP2 and VP3 capsid proteins (Clone B1, Progen Immunodiagnostika, Heidelberg, Germany) were used, respectively. A goat anti-mouse-HRP (Jackson Laboratories, Bar Harbor, ME, USA) was used as a secondary antibody, followed by detection using ECL Western blotting detection reagents (Amersham Pharmacia Biotech).

Determination of plasma cholesterol levels

Plasmas from tail-vein bleeds were diluted 1/10 in PBS and 10 μl was used to estimate total cholesterol levels by the infinity cholesterol reagent assay system (Sigma), as described by the manufacturer. Briefly, the assay was performed in microtitre 96-well plates and 90 μl of infinity cholesterol reagent per sample was used followed by an incubation at 37°C for 5 min and then measuring the absorbance at 510 nm.

Plasma lipoprotein distribution analysis

Lipoprotein profiles of pooled plasma (2 μl) was performed by electrophoresis on precast alkaline buffered (pH 8.8) agarose gels (YSI, Farnborough, UK) followed by staining with a lipid-specific Sudan black stain according to the manufacturer's instructions. The concentrations of the Sudan black-stained lipoprotein fractions were determined by scanning densitometry using an imaging densitometer model GS-670 with Molecular Analyst Version 1.4 software (BioRad).

Dissection and examination of aorta for atherosclerotic lesions

Following the death of the animals, the hearts and thoracic aortas were taken and any adventitial fat was dissected away before cutting the aortas longitudinally and then pinning them out *en face* on to cork beds. The dissected aortas were stained with Oil-Red-O stain (Sigma) modified from previously described methods.[22,23] Briefly, the aortas were fixed in PBS/4% formaldehyde for 3 days, washed in PBS followed by staining with 1.8% Oil-Red-O in 60% isopropanol for 15 min at room temperature, then destained in 60% isopropanol for 5 min. The stained aortas were transferred to PBS for storage at 4°C. Images of the aortas were captured with a Nikon digital camera and analysis of aortic lesion area from the aortic root down to the diaphragm, including the aortic arch, was achieved using the image analysis software Sigma Scan Pro5.

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Paradoxical reduction of atherosclerosis in apoE-deficient mice with obesity-related type 2 diabetes

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Abstract

Objective: The effect of obesity and insulin resistance on the development of atherosclerosis was evaluated in apoE-deficient ($ApoE^{-/-}$) mice. A previously described obesity model, in which the hypothalamic satiety center can be destroyed by a single gold thioglucose (GTG) injection, was used. To evaluate the effect of starvation on atherosclerosis $ApoE^{-/-}$ mice were food-restricted with 25% less chow than ad libitum-fed control mice. **Methods:** Sixty-eight $ApoE^{-/-}$ mice were allocated into a control group ($n=20$), a GTG-injected group ($n=28$), and a food-restricted group ($n=20$). The control and food-restricted mice were injected with suline instead of GTG. The control and GTG-injected mice had free access to food, and all mice had free access to water during the study period. **Results:** After 4 months, the GTG-injected mice were significantly overweight (mean body weight (g): 33±2.11 vs. 23±0.24 and 17±0.31 in control and food-restricted mice, respectively), obese, hypertriglyceridemic, insulin-resistant, hyperinsulinemic (mean plasma insulin (ng/ml): 2.45 and 0.43 in obese and control mice, respectively), and hyperglycemic (mean plasma glucose (mmol/l): 11.03 and 7.80 in obese and control mice, respectively). Unexpectedly, these obese and diabetic mice developed significantly less atherosclerosis compared with lean non-diabetic control mice. Food-restricted mice also developed less atherosclerosis compared to control mice. **Conclusions:** These findings may question the usefulness of mouse models in studying the relation of obesity-related type 2 diabetes to atherosclerosis and also the relevance of results obtained in $apoE^{-/-}$ mice with reduced weight gain during intervention.

Keywords: Atherosclerosis; Type 2 diabetes; Obesity; Insulin resistance; Gold thioglucose; Food restriction; Apolipoprotein E deficient mice

1. Introduction

Type 2 diabetes is characterized by obesity, insulin resistance; hyperinsulinemia, hyperglycemia and dyslipidemia; in combination with essential hypertension also referred as the metabolic syndrome. Type 2 diabetes constitutes an increasing health problem world wide, mainly because of the increasing prevalence of obesity and reduced physical activity [1]. Myocardial infarction, stroke and peripheral vascular disease cause 80% of all diabetic mortality with coronary artery disease as the most frequent cause of death [1]. The overall risk of cardiovascular disease is increased two to four times in patients with type 2 diabetes [2]. Even after adjusting for hypertension and hypercholesterolemia, diabetes in itself remains as an independent risk factor for the development of cardiovascular disease [3,4]. The specific interaction between type 2 diabetes and atherothrombotic disease is, however, poorly understood, partly because of the lack of appropriate animal models.

Therefore, the aim of the present study was to create an animal model in which obesity-induced type 2 diabetes and atherosclerosis coexist in such a way that the effect of diabetes and its metabolic components on atherosclerosis could be examined. On standard chow diet, the hypercholesterolemic $apoE^{-/-}$ mouse develops advanced atherosclerosis spontaneously [5]. The wild-type counter-

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part, the C57BL mouse, becomes obese and insulin-resistant by a single GTG injection, which destroys the hypothalamic neurons regulating food intake and energy expenditure [6]. Therefore, an animal model of innate susceptibility to atherosclerosis with acquired type 2 diabetes superimposed might be created in apoE$^{-/-}$ mice by a single GTG injection.

2. Methods

2.1. Mice, GTG and food restriction

A total of 70 female apoE$^{-/-}$ mice, backcrossed for 10 generations on the C57BL/6J background (M&B, Ry, Denmark), were fed regular pelleted chow (Altromin 1324) with a fat content of 5% (w/w) throughout the study. At 8 weeks of age, the mice were injected i.p. with 0.5 mg GTG (Sigma–Aldrich, Vallensbaek, Denmark) per g body weight (n=30) or saline (n=40). Two died shortly after GTG-injection, leaving 28 mice in the GTG group. The mice receiving saline were allocated into a control group (n=20), and a food-restricted group (n=20) that was given 25% less food than that eaten by control mice. Control and GTG-injected mice had free access to food, and all mice had free access to water. The mice were killed 4 months later at the age of 6 months. The mice were maintained five per cage in a temperature-controlled (21 °C) facility with a strict 12-h light/dark cycle. Body weight and food consumption were measured throughout the study. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the mice were housed and cared for according to national guidelines, and the National Animal Ethics Committee approved all the procedures.

2.2. Plasma insulin and glucose

Three weeks before the end of the study, 5-h fasting blood samples (100 μl, obtained between 10:00 and 12:00 h) were drawn from the retro orbital venous plexus of non-anesthetized mice into chilled tubes containing heparin/aprotinin (Løvens Kemiske Fabrik, Ballerup, Denmark). The blood was centrifuged, and plasma was stored immediately at −20 °C. Glucose was determined by the glucose-oxidase method (GOD/PAP, Boehringer-Mannheim, Mannheim, Germany). Insulin was measured using a sensitive rat insulin kit (Linco Res., MO, USA).

2.3. Insulin sensitivity test

One week before the end of the study, an intraperitoneal insulin sensitivity test (IST) was performed in non-anesthetized mice fasted for 18 h. Blood glucose levels were determined in whole blood (HemoCue AB, Ängelholm, Sweden) obtained by cutting the tip of the tail (t=0). Then, insulin (Actrapid; Novo Nordisk, Gentofte, Denmark) in a dosage of 1 U per kg body weight diluted in 0.9% sterile NaCl containing bovine serum albumin (g/ml) (fatty acid free; Boehringer-Mannheim) was injected i.p. Exactly 30 min later (t=30), blood glucose levels were determined again. The glycemic response to the weight-adjusted dose of insulin, expressed as the percentage fall in glucose ($[\text{glucose}]_{t=0} - [\text{glucose}]_{t=30}/[\text{glucose}]_{t=0} \times 100\%$) was used as an insulin sensitivity marker. After the insulin sensitivity test 100 mg glucose was injected i.p. in order to reduce hypoglycemia.

2.4. Lipids and lipoproteins

At the end of the study, non-fasting plasma levels of total cholesterol (TC) and triglycerides (TG) were measured enzymatically on a Cobas Fara analyzer using reagents from Roche Diagnostica (Copenhagen, Denmark). Lipoprotein cholesterol distributions were evaluated in individual samples from five mice in each group after fractionation by size exclusion chromatography as previously described [7].

2.5. Quantification of atherosclerosis

At 6 months of age (4-month study period) the mice were anesthetized with pentobarbital (5 mg/ml) and exsanguinated by withdrawing the maximum amount of blood from the right ventricle. The blood was centrifuged, and plasma was stored at −20 °C for later lipid measurements. The mice were flushed with isotonic saline and then perfusion-fixed (phosphate-buffered 4% formaldehyde, pH 7.2) at ~100 mmHg via the left ventricle and immersed in the fixative for 6 h. The heart, including the aortic root, was removed and cut transversely [8], and embedded in paraffin. The aortic root was cross-sectioned serially at 4-μm intervals. Five sections taken at 80-μm intervals, spanning 320 μm of the aortic root from the commissures of the aortic leaflets and outward, were stained with orcein (for elastic tissue) and evaluated microscopically. Plaque area, delimited by the lumen and the internal elastic membrane, was measured blindly by the same person (LGL) using computer-assisted image analysis (Sigma Scan Pro, San Rafael, CA, USA), and the mean of the five measurements was determined (mean plaque area).

2.6. Obesity

Four sets of fat pads (inguinal, periovarial, renal and retroperitoneal fat pads) were dissected and weighed. The renal and the retroperitoneal fat pads were taken out in one piece. An adiposity index (AI) was computed for each mouse (Σ(fat pads)/body weight)×100%).

2.7. Statistical analysis

Values are reported as mean±S.E.M. The Kruskal–Wallis test (non-parametric test) was used to determine differences between means of the three groups of mice. A non-parametric correlation test, Spearman's rho (r_s), was used to determine correlations between the different parameters. The Mann–Whitney *U*-test or the independent *t*-test was used to compare the groups pair-wise. $P<0.05$ was considered statistically significant. The statistical analysis was done using the SPSS-software version 10.0 for Windows (Chicago, IL, USA).

3. Results

Of the 68 mice that entered the study, 14 mice (three controls (15%), five food-restricted (25%) and six GTG-injected (21%)) died during or immediately after blood sampling (5-h fasting values). Body weight did not differ between those mice who died and those who survived (control $P=0.11$, food-restricted $P=0.65$, and GTG-injected $P=0.15$).

3.1. Body weight, food consumption and obesity

The mean body weight in the three groups did not differ at baseline ($P=0.31$) but did at the end of the study ($P<0.0001$, Fig. 1). The GTG-injected mice were divided into two groups, responders and non-responders, according to their weight gain. Responders ($n=12$) were characterized by an extraordinary long-term weight gain resulting in a final body weight and AI markedly larger than those of control mice (bodyweight and AI>mean of controls+ 5S.D.) (Figs. 1, 2A,B). The non-responding GTG-injected mice remained lean ($n=10$) and followed a growth curve similar to that of control mice (Fig. 1). Nearly all GTG-injected mice experienced a weight loss during the first week after the injection. However, the mean weight loss was larger in the responding mice than in the non-responders (4.0±0.7 vs. 1.8±1.9 g, $P=0.0025$).

A marked reduction in food consumption was seen in the first week after GTG injection (Fig. 3). Thereafter, food intake increased rapidly to a level similar to control mice. Throughout the rest of the study the consumption curves of the control and GTG-injected mice did not differ ($P=0.87$). Measurable hyperphagia was not observed in cages containing GTG-injected mice. It should, however, be noted that non-responding and responding mice were housed together.

3.2. Glucose, insulin, and glycated hemoglobin

GTG-injected mice had significantly higher 5-h fasting glucose and insulin values than control and food-restricted mice ($P<0.0001$, Table 1). Body weight and AI correlated positively and significantly with fasting insulin ($n=22$, $r_s=0.60$ (weight), $r_s=0.55$ (AI), $P<0.001$) and fasting glucose ($n=22$, $r_s=0.53$ (weight), $r_s=0.56$ (AI), $P<0.001$).

Between control and food-restricted mice, fasting glucose ($P=0.24$) and insulin ($P=0.48$) did not differ. Glycated hemoglobin was similar in all groups (data not shown).

3.3. Insulin sensitivity test

The glycemic response to i.p. injection of a weight-adjusted dose of insulin differed significantly between groups ($P<0.0001$, Table 1). A less and more pronounced

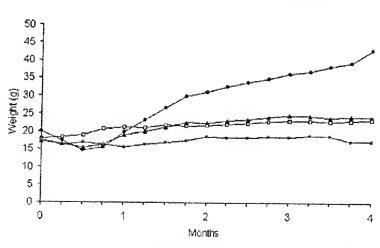

Fig. 1. Body weight in apoE[illegible] mice throughout the 4 months study period. The final body weight of the responding GTG-injected, non-responding GTG-injected, control and food restricted mice were 40.6±6.9, 24±2.1, 23.4±0.24 and 17.1±0.31 g, respectively ($P<0.001$)

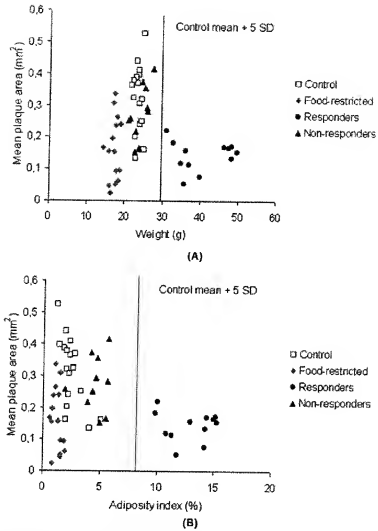

Fig. 2. (A) Atherosclerotic plaque area as a function of body weight. (B) Atherosclerotic plaque area as a function of adiposity index (AI). The AI of the responding GTG-injected, non-responding GTG-injected, control and food-restricted mice were 12.9, 4.5, 2.4 and 1.2%, respectively ($P<0.0001$). Those GTG injected mice that gained weight more than control mean±SS.D. were exactly the same as those with an AI larger than control mean±SS.D.

glucose lowering response was seen in GTG-injected mice (indicating insulin resistance) and food-restricted mice (indicating insulin hypersensitivity), respectively, compared to control mice (Table 1). No mice died during the procedure.

3.4. Plasma lipids and lipoproteins

The plasma TC levels in the food-restricted, control and GTG group were 16.9±0.57, 17.3±0.88 and 19.8±0.88 mmol/l, respectively, and these concentrations differed significantly ($P=0.013$). For the obese mice the plasma TC correlated positively and significantly with body weight ($n=22$; $r_s=0.55$, $P=0.034$). The plasma TG levels in the food-restricted, control and GTG group were 0.8±0.2, 0.58±0.16 and 1.1±0.39 mmol/l, respectively ($P=0.005$).

Lipoprotein-cholesterol distributions were determined by size exclusion chromatography (Fig. 4). The VLDL- and LDL-cholesterol peaks were higher in GTG-injected mice and lower in food-restricted mice compared with control mice. There was, however, no significant difference between the lipoprotein distributions of the three groups. The HDL-cholesterol was barely detectable in any of the groups.

3.5. Atherosclerosis

Atherosclerosis was evaluated in the aortic root. Ad-

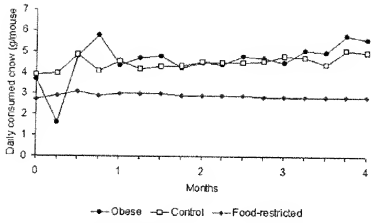

Fig. 3. Mean food-consumption per mouse per day. The obese and the control group were fed ad libitum, whereas the lean mice were strictly kept at a daily amount of food (25% food reduction compared to the control).

vanced atherosclerotic plaques were observed in all groups. No plaque rupture or luminal thromboses were seen.

Mean plaque area differed significantly between the three groups of mice ($P=0.005$, Fig. 5), despite a substantial overlap in plaque area among the groups. Compared to control mice, GTG-injected mice ($P=0.004$) and food-restricted mice ($P<0.0001$) had smaller plaques. The smaller plaque area in the GTG-injected mice was only seen in responders (Fig. 5). There was no relationship between the initial weight loss and plaque size in responders, nor in non-responders (data not shown). The mean plaque area was similar in GTG-injected and food-restricted mice ($P=0.25$, Fig. 5).

In GTG-injected mice, mean plaque area correlated negatively with body weight ($n=22$, $r_s=-0.55$, $P=0.013$; Fig. 2A), obesity (AI) ($n=22$, $r_s=-0.67$, $P=0.003$; Fig. 2B) and fasting insulin level ($n=22$, $r_s=-0.38$, $P=0.022$).

Table 1
Fasting plasma values of insulin and glucose and the results of insulin sensitivity test (IST): values of insulin and glucose were obtained 3 weeks before the end of the study on 5-h fasting blood samples; IST was performed 1 week before the end of the study in non-anesthetized mice fasted for 18 h

	Insulin* (ng/ml)	Glucose* (mmol/l)	IST* (%)
Food-restricted ($n=15$)	0.39±0.05	7.35±0.26	0.71±0.03
Control ($n=17$)	0.43±0.04	7.80±0.26	0.61±0.03
GTG ($n=22$)	1.85±0.34	10.1±0.37	0.47±0.02
Non-responders ($n=10$)	1.12±1.37	9.03±1.44	0.51±0.10
Responders ($n=12$)	2.45±1.58	11.03±1.4	0.44±0.10

Data are presented as mean±S.E.M.
*$P<0.001$ (comparison of the mean differences between the three groups: GTG (responders+non-responders), control and food-restricted).

4. Discussion

This study shows for the first time that GTG injection in atherosclerosis-prone apoE$^{-/-}$ mice induces obesity, insulin resistance, hyperinsulinemia, hyperglycemia and hypertriglyceridemia, i.e., type 2 diabetes mellitus. Unexpectedly, these obese and diabetic mice developed significantly less atherosclerosis than the lean non-diabetic control mice. The food-restricted mice also developed less atherosclerosis.

The neurotoxic glucose analogue GTG has been used for decades to induce obesity in mice [6]. Just a single peripheral GTG injection destroys within 24 h leptin receptor-positive hypothalamic neurons, including those regulating food intake (satiety) and energy expenditure (metabolic rate and heat production), resulting in subsequent development of obesity, leptin resistance (hyperleptinemia) and insulin resistance (hyperinsulinemia)-features that also characterize human obesity [9–12]. An acute weight loss is usually seen during the first week after GTG injection but most of the responding mice will be obese, hyperleptinemic and hyperinsulinemic just 1 month later [9,10]. We observed an increase in body weight in our responding apoE$^{-/-}$ mice. They were severely obese, hyperinsulinemic and hyperglycemic 4 months after the GTG injection. Similarly, Hirano et al. recently reported that plasma insulin, glucose and cholesterol levels were elevated by 213, 23, and 51%, respectively, in obese apoE$^{-/-}$ mice 14 weeks after GTG injection [13].

In healthy apoE$^{-/-}$ mice, no major fluctuations are seen in plasma glucose, insulin, cholesterol and triglyceride

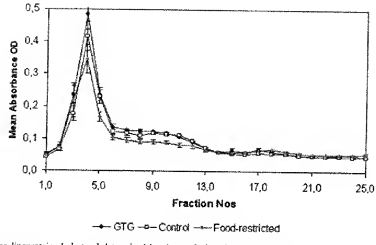

Fig. 4. Distribution of plasma lipoprotein cholesterol determined by size exclusion chromatography. Points represent the mean of five chromatographic separations that were performed on plasma from five mice in each group, and the bars represent S.E.M.

levels between 2 and 6 months of age [5,14]. However, no such longitudinal data are available for GTG-treated apoE$^{-/-}$ mice. With the contrasting result in mind (less atherosclerosis despite a seemingly proatherogenic metabolic profile), it would be interesting to know the temporal changes in the glucose and lipid metabolism after GTG-injection.

Most of the non-responders experienced a weight loss immediately after GTG injection. However, the average weight loss of the non-responding GTG-injected mice was less than that observed by the responding mice (1.7 vs. 4.0 g). It seems unlikely that such a short-term weight loss in adult mice protects against subsequent long-term development of atherosclerosis. The lack of correlation between the initial weight loss and plaque size in responders and in non-responders argues against this possibility.

Non-responding GTG-injected mice and control mice developed similar amount of atherosclerosis and significantly more than responders. A systemic obesity-unrelated anti-atherogenic effect of GTG such as regulation of blood pressure therefore seems unlikely. However, the blood GTG levels might have been higher in responders than in

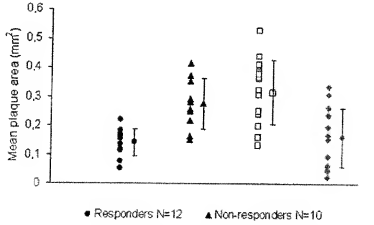

Fig. 5. Atherosclerosis measured in the aortic root (mm^2). Mean plaque area (mm^2) was 0.14±0.05, 0.28±0.09, 0.32±0.11 and 0.19±0.12 in the responding GTG-injected, non-responding GTG-injected, control and food restricted mice, respectively. Mean plaque area±1 S.D. is shown for each group ($P < 0.005$).

non-responders, reflecting the extent of the immediate weight loss and suggesting that GTG is highly toxic and affects the mice in general. However, other effects of GTG such as regulation of blood pressure and other systemic effects have not been described previously. The destruction of hypothalamic neurons is the only known action of GTG [6,11,15].

GTG-induced obesity is usually caused by an increase in energy intake and a decrease in energy expenditure. However, the latter is enough to induce obesity. Pair-feeding experiments of GTG-injected mice have shown that obesity develops without hyperphagia [10,12]. This together with the collective housing of responders and non-responders may explain that hyperphagia was not documented in the GTG-injected mice.

The apoE$^{-/-}$ mouse is a well-established animal model in atherosclerosis research. On standard chow, apoE$^{-/-}$ mice develop severe hypercholesterolemia and advanced atherosclerotic lesions spontaneously throughout the arterial system [5,16]. A high-fat Western-type diet may double the plasma cholesterol level and accelerate the development of atherosclerosis. However, minor changes in total cholesterol and triglyceride levels, as those seen in the present study, have no documented (anti)-atherogenic impact in this model [17].

The apoE$^{-/-}$ mice, used in the present study, were backcrossed 10 generations into the C57BL/6 background, which is the most used mouse strain in studies determining the relation between obesity and insulin resistance [18–20]. In C57BL/6 mice, diets high in fat [18–20] and/or simple carbohydrates [17] easily induce obesity and insulin resistance. However, using such a dietary approach, we were not able to induce either obesity or insulin resistance in our apoE$^{-/-}$ mice, despite their C57BL/6 genetic background (unpublished data). Nevertheless, the present study documents that the apoE$^{-/-}$ mouse is not inherently resistant to diet-induced obesity and insulin resistance, because both conditions are prone to develop, even in chow-fed mice, after GTG injection. The relevance of this observation is strengthened by the positive and highly significant correlation between obesity and markers of insulin resistance (fasting hyperinsulinemia and hyperglycemia) in the GTG-injected mice.

The paradoxical finding of reduced atherosclerosis in obese apoE$^{-/-}$ mice with type 2 diabetes is, however, in agreement with corresponding results obtained in other mouse models. Several genetic models of obesity-linked insulin resistance and type 2 diabetes exist in mice, among others obese (*ob*), diabetic (*db*), fat (*fat*), tubby (*tub*), lethal yellow (A^y), and KKA^y mice. Atherosclerosis does not develop spontaneously in any of these mice, and fatty streak formation in the aortic root is not accelerated, compared to their wild-type counterpart, by an atherogenic diet high in fat, cholesterol, and cholate [21]. Most of these genetically obese and insulin-resistant mice strains develop in fact significantly less atherosclerosis on the atherogenic diet than their lean wild-type controls. Also the LDL receptor-deficient mouse appears to develop less atherosclerosis with diet-induced insulin-resistance, documented by less atherosclerosis in obese, insulin-resistant mice fed high-fat diet than in lean, non-insulin-resistant, fructose-fed mice [22]. Thus, obesity and insulin resistance acquired or of genetic origin, appears to confer relative protection against the development of atherosclerosis in mice.

The reason for this paradoxical protection against atherosclerosis in insulin-resistant mice (versus some strains of rats [23]) is unknown, but an unusual lipoprotein pattern might play a critical role [24]. Insulin resistance and type 2 diabetes are associated with an extremely atherogenic dyslipoproteinemia in humans (high VLDL, low HDL and small dense LDL-particles) but the opposite might theoretically occur with insulin resistance and type 2 diabetes in mice caused by their characteristic non-human-like lipoprotein metabolism. Triglyceride-rich VLDL particles may be so large that they are unable to penetrate into the arterial wall resulting in reduced atherosclerosis, whereas smaller cholesterol-rich particles (IDL/LDL) are much more atherogenic. This probably explains the atheroprotective effect of alloxan-induced type 1 diabetes observed in cholesterol fed rabbits [25]. In the present study, however, there was no change in lipoprotein-cholesterol distribution that could explain the lesser amount of atherosclerosis observed in our obese mice with type 2 diabetes.

Compared with ad libitum feeding, 40% food restriction increases longevity substantially in mice [26]. In rabbits food restriction does not lead to any regression of atherosclerosis [27]. Only a few studies have addressed the effect of food restriction on the development of atherosclerosis in mice despite the fact that many drug regiments claimed to be atheroprotective in mice also inhibit normal growth [28–30].

In the present study, 25% food restriction alone reduced the development of atherosclerosis significantly in apoE$^{-/-}$ mice compared with those fed chow ad libitum. This anti-atherogenic effect of food restriction could not be explained by beneficial changes in plasma total cholesterol and triglyceride levels. The same was observed in a newly published study [31], which also raises the hypothesis that the atheroprotective effect of food restriction is linked to reduced oxidative stress in the arterial wall [31]. Although the anti-atherogenic effect of restricted feeding is not necessarily seen with reduced caloric intake caused by a drug, it should be considered as a possibility when an anti-atherogenic intervention also retards or prevents the normal growth of a mouse.

A limitation of the study is that we used only female mice. The advantage of using female mice is that they develop larger atherosclerotic plaques in the aortic root than male mice [32]. However, the opposite might be true when the total arterial tree is examined [33]. GTG is

shown to have equal effect in male as well as in female mice [9,34] so in that matter we have no reason to believe that our results would have been different using male mice.

5. Conclusions

GTG-injected ad libitum fed apoE$^{-/-}$ mice developed type 2 diabetes, including obesity, insulin resistance, hyperinsulinemia, hyperglycemia and hypertriglyceridemia. Contrary to our expectations, these obese and diabetic mice developed less atherosclerosis than lean non-diabetic control mice. Food-restricted mice also developed less atherosclerosis. These findings may question the usefulness of mouse models in studying the relation of obesity and/or type 2 diabetes to atherosclerosis, and the relevance of results obtained in apoE$^{-/-}$ mice with reduced weight gain during intervention.

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Impaired Development of Atherosclerosis in Abcg1 / Apoe / Mice: Identification of Specific Oxysterols That Both Accumulate in Abcg1 / Apoe / Tissues and Induce Apoptosis

Elizabeth J. Tarling, Dragana D. Bojanic, Rajendra K. Tangirala, Xuping Wang, Anita Lovgren-Sandblom, Aldons J. Lusis, Ingemar Bjorkhem and Peter A. Edwards

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Impaired Development of Atherosclerosis in *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ Mice

Identification of Specific Oxysterols That Both Accumulate in *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ Tissues and Induce Apoptosis

Elizabeth J. Tarling, Dragana D. Bojanic, Rajendra K. Tangirala, Xuping Wang, Anita Lovgren-Sandblom, Aldons J. Lusis, Ingemar Bjorkhem, Peter A. Edwards

Objective—To generate *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ mice to understand the mechanism and cell types involved in changes in atherosclerosis after loss of ABCG1.

Methods and Results—ABCG1 is highly expressed in macrophages and endothelial cells, 2 cell types that play important roles in the development of atherosclerosis. *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice and recipient *Apoe*$^{-/-}$ mice that had undergone transplantation with bone marrow from *Apoe*$^{-/-}$ or *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ mice were fed a Western diet for 12 or 16 weeks before quantification of atherosclerotic lesions. These studies demonstrated that loss of ABCG1 from all tissues, or from only hematopoietic cells, was associated with significantly smaller lesions that contained increased numbers of TUNEL- and cleaved caspase 3–positive apoptotic *Abcg1*$^{-/-}$ macrophages. We also identified specific oxysterols that accumulate in the brains and macrophages of the *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ mice. These oxysterols promoted apoptosis and altered the expression of proapoptotic genes when added to macrophages in vitro.

Conclusion—Loss of ABCG1 from all tissues or from only hematopoietic cells results in smaller atherosclerotic lesions populated with increased apoptotic macrophages, by processes independent of ApoE. Specific oxysterols identified in tissues of *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ mice may be critical because they induce macrophage apoptosis and the expression of proapoptotic genes. ***(Arterioscler Thromb Vasc Biol.* 2010;30:1174-1180.)**

Key Words: ABCG1 ■ apolipoprotein E ■ atherosclerosis ■ apoptosis ■ Bid ■ Bok ■ oxysterols

The ATP binding cassette transporter, subfamily G, member 1 (ABCG1) is 1 member of a large superfamily of membrane proteins that function to transport substrates across specific membranes.[1,2] Studies[3–7] with *Abcg1*$^{-/-}$ β-galactosidase (LacZ) knock-in mice have demonstrated that ABCG1 is expressed in numerous organs and cell types, with particularly high expression in macrophages, endothelial and epithelial cells, and neurons.

Numerous studies have shown that ABCG1 can function to efflux cholesterol and/or other sterols from cells to various exogenous acceptors, including high-density lipoprotein.[8] Studies[3,6,9,10] with *Abcg1*$^{-/-}$ mice demonstrated that pulmonary macrophages accumulate massive levels of cholesterol and sterol esters, consistent with these cells being particularly sensitive to loss of function of this transporter. These lipid-loaded *Abcg1*$^{-/-}$ pulmonary macrophages also undergo increased apoptosis.[11] These data are consistent with the normal role of pulmonary macrophages in clearing cholesterol-containing surfactant from the extracellular space,[12] with ABCG1 then functioning to eliminate the sterols from the cells to maintain cellular sterol homeostasis.

Loss of ABCG1 from macrophages results in increased expression of multiple inflammatory genes, consistent with a stimulatory effect of the accumulating cellular sterols on inflammation.[9,11,13] Earlier studies demonstrated that endothelial cells, like macrophages, express particularly high levels of ABCG1.[14] Interestingly, the administration of a Western diet to *Abcg1*$^{-/-}$ mice was recently shown to increase the inflammatory status and sterol levels of endothelial cells.[13] Taken together, these data suggest that loss of ABCG1 results in subtle or gross changes in cellular sterols that may result in induction of inflammatory genes and/or increased apoptosis in 2 cell types (endothelial cells and macrophages) that are known to play critical roles in the development of atherosclerosis.

Atherosclerosis is a complex disease that is characterized in the early stages by the accumulation of lipid-loaded macrophages (foam cells) in the intima.[15] The initial findings

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that loss of ABCG1 led to the accumulation of sterol-loaded macrophages in the lungs of *Abcg1*$^{-/-}$ mice[3,6,10,11] suggested that hyperlipidemic mice lacking functional ABCG1 would exhibit accelerated atherosclerosis. However, *Abcg1*$^{-/-}$ mice have a normal plasma lipoprotein profile and, thus, do not develop significant atherosclerotic lesions even when fed a Western diet.[16] To assess the role of ABCG1 in the development of atherosclerosis, 3 groups independently performed bone marrow transplantation studies using donor cells from wild-type or *Abcg1*$^{-/-}$ C57BL/6 mice and recipient hyperlipidemic low-density lipoprotein receptor null (*Ldlr*$^{-/-}$) mice. Although the protocols used in these studies were similar, the conclusions were not; one group reported that transplantation of *Abcg1*$^{-/-}$ bone marrow led either to a modest but significant increase in atherosclerotic lesions in the *Ldlr*$^{-/-}$ recipient mice[6,17] or to no change in lesion size.[17,18] In contrast, Baldan et al[16] and Ranalletta et al[19] observed a significant decrease in lesion size in *Ldlr*$^{-/-}$ mice receiving *Abcg1*$^{-/-}$ cells. It remains to be determined whether these inconsistencies result from differences in the genetic backgrounds of the mice, from different concentrations of cholesterol in the diet, or from different treatment times that affect lesion progression.

Alternative mechanisms, which are not necessarily exclusive, were invoked to explain the unexpected decrease in lesion size noted in 2 of the transplantation studies.[16,19] Ranalletta et al[19] proposed that the *Abcg1*$^{-/-}$ macrophages secreted increased amounts of ApoE protein, a known antiatherogenic protein. It was also suggested that increased expression of a second sterol transporter, ABCA1, in the *Abcg1*$^{-/-}$ macrophages might reduce sterol accumulation in the foam cells and, thus, impair lesion development.[19] In contrast, Baldan et al[16] proposed that the smaller lesions were a result of increased apoptosis of the *Abcg1*$^{-/-}$ macrophages that populated the atherosclerotic lesions of *Ldlr*$^{-/-}$ mice. A role for ABCG1 in protection against apoptosis is consistent with studies showing that the lungs of *Abcg1*$^{-/-}$ mice contain increased TUNEL-positive apoptotic cells[11] and that overexpression of ABCG1 in cultured cells attenuates oxysterol-induced cell death, possibly by stimulating the efflux of either 7β-hydroxycholesterol[20] or 7-ketocholesterol[21] to exogenous high-density lipoprotein.

Apoptosis plays an important role in the development of atherosclerotic lesions.[22–24] An increase in macrophage apoptosis in early lesions has been associated with decreased lesion progression.[25] In contrast, an increase in macrophage apoptosis in advanced lesions is thought to promote the development of the necrotic core, a key factor in vulnerable plaque formation and acute thrombosis.[24] The increase in apoptotic cells in lesions may result from the accumulation of unesterified cholesterol and/or oxysterols because these lipids are known to stimulate proapoptotic processes.[25,26] Support for a role for ABCG1 in preventing apoptosis[16] came from studies[16,27] showing that *Abcg1*$^{-/-}$ or *Abcg1*$^{-/-}$ *Abca1*$^{-/-}$ macrophages exhibit increased apoptosis in vitro compared with wild-type cells after a challenge with oxidized low-density lipoprotein (oxLDL).

We report that hyperlipidemic apolipoprotein E null (*Apoe*$^{-/-}$) mice lacking ABCG1 in all tissues or in hematopoietic cells only exhibit decreased lesions, decreased aortic lesion calcification, and increased macrophage apoptosis as a result of the accumulation of specific proapoptotic oxysterols.

Methods

We fed a Western diet for 12 or 16 weeks to *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice and to recipient *Apoe*$^{-/-}$ mice that had undergone transplantation with bone marrow from *Apoe*$^{-/-}$ or *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ mice. Atherosclerotic lesion size, apoptosis, and oxidized sterol concentrations in macrophages in these mice were determined. For details and further methods, please see the Supplemental Material (available online at http://atvb.ahajournals.org).

Results

Characterization of *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ Mice

Endothelial cells of *Abcg1*$^{-/-}$ mice fed a Western diet accumulate 7-ketocholesterol, a nonenzymatic product of cholesterol autoxidation.[13] *Abcg1*$^{-/-}$ mice also exhibit decreased endothelium-dependent vasodilatation and decreased endothelial nitric oxide synthase activity.[13] Macrophages from *Abcg1*$^{-/-}$ mice also exhibit increased expression of inflammatory genes and accumulate intracellular 7-ketocholesterol.[9,11] In addition, loss of ABCG1 from macrophages has been reported to result in increased secretion of ApoE protein,[19] an antiatherosclerotic protein.[28]

Consequently, to better understand the effect of loss of function of ABCG1 from all cell types, including macrophages and endothelial cells, and to remove any confounding effects that could arise from altered secretion of ApoE from macrophages, we generated *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ double-knockout (DKO) mice. Analysis of the plasma showed that, compared with *Apoe*$^{-/-}$ mice, DKO mice had increased hemoglobin (14.00±0.67 versus 12.68±0.96; $P<0.04$; mean±SEM n=10) and hematocrit (40.42±1.94 versus 36.80±2.14; mean±SEM, n=10; $P<0.03$) values; however, lipid and lipoprotein levels and a broad array of hematology values did not significantly differ between the 2 genotypes (data not shown).

To accelerate the development of atherosclerosis, DKO and *Apoe*$^{-/-}$ mice were challenged with a Western diet (21% fat and 0.2% cholesterol). After 16 weeks, analysis of plasma from DKO and *Apoe*$^{-/-}$ mice indicated that there was no significant difference in lipid levels (Supplemental Table I) or the lipoprotein profile (Supplemental Figure IA). Lungs of the DKO, but not the *Apoe*$^{-/-}$ mice, appeared white and stained positive with oil red O, especially in areas enriched in LacZ-expressing macrophages (Supplemental Figure II; data not shown). In addition, the red and white pulp of spleens of the DKO, but not *Apoe*$^{-/-}$ mice, contained cells that were positive for both LacZ and oil red O (Supplemental Figure II). Thus, multiple tissues of the DKO mice exhibited evidence of neutral lipid accumulation.

Abcg1$^{-/-}$ *Apoe*$^{-/-}$ Mice Have Decreased Atherosclerotic Lesions Containing Increased Numbers of Apoptotic Macrophages

After 16 weeks on the Western diet, atherosclerotic lesions were determined both by en face analysis of the Sudan IV–stained descending aorta or after analysis of stained frozen sections (25 to 30 sections per mouse) of the aortic root

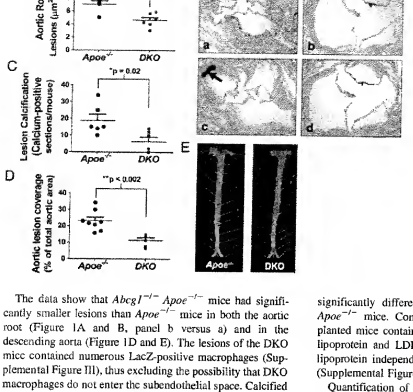

Figure 1. Atherosclerosis and lesion calcification are reduced in *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ compared with *Apoe*$^{-/-}$ mice. DKO and *Apoe*$^{-/-}$ mice (6 to 8 mice per group) were fed a Western diet for 16 weeks. A, Frozen sections (25 to 30 sections per mouse; n=6 mice per group) from the aortic root were stained with oil red O and counterstained with hematoxylin and fast green before lesion areas were determined, as described in the "Methods" section. Each point represents an individual mouse. **P<0.01. B, Representative oil red O–stained sections counterstained with hematoxylin and fast green (a and b) and adjacent sections stained with von Kossa (c and d). Calcification/calcium phosphate deposits are indicated by arrows in panels a and c. C, Quantification of lesion calcification. *P<0.05. D, Lesions in the descending aorta were identified by en face analysis and quantified as described in the "Methods" section (n=8 mice per group). Data represent mean±SEM. **P<0.01. E, Representative Sudan IV–stained aortas are shown from the 2 genotypes.

The data show that *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ mice had significantly smaller lesions than *Apoe*$^{-/-}$ mice in both the aortic root (Figure 1A and B, panel b versus a) and in the descending aorta (Figure 1D and E). The lesions of the DKO mice contained numerous LacZ-positive macrophages (Supplemental Figure II), thus excluding the possibility that DKO macrophages do not enter the subendothelial space. Calcified deposits in the lesions of the aortic root, identified after staining of sections with either von Kossa or oil red O, hematoxylin, and fast green, were significantly reduced in the *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ mice (Figure 1C; and Figure 1B, panel d versus c and b versus a), consistent with the smaller lesions in the DKO mice. Photomicrographs taken at a higher magnification illustrate that the calcium deposition occurs within the aortic lesions, adjacent to the medial layer (Supplemental Figure IV).

Loss of ABCG1 From Hematopoietic Cells Delays the Development of Atherosclerosis and Increases Apoptotic Macrophages in the Lesions, Independent of ApoE

To determine the relative importance of hematopoietic and nonhematopoietic *Abcg1*$^{-/-}$ cells on the observed changes in atherosclerosis noted in the *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ mice (Figure 1), we performed bone marrow transplantation studies in which bone marrow from either *Apoe*$^{-/-}$ or DKO mice was transplanted into recipient *Apoe*$^{-/-}$ animals. After a 4-week recovery period, the mice were fed a Western diet for 12 weeks. An analysis of the lungs of the *Apoe*$^{-/-}$ recipients indicated that mice that had been transplanted with DKO donor cells, but not those receiving *Apoe*$^{-/-}$ cells, contained LacZ-positive cells and white patches consistent with lipid deposition in macrophages (data not shown).

Neither plasma lipid levels (Supplemental Table II) nor plasma lipoprotein profiles (Supplemental Figure IB) were significantly different between the 2 groups of recipient *Apoe*$^{-/-}$ mice. Compared with wild-type mice, all transplanted mice contained elevated levels of very low-density lipoprotein and LDL and decreased levels of high-density lipoprotein independent of the genotype of the donor cells (Supplemental Figure 1B; data not shown).

Quantification of the atherosclerotic lesions showed that they were significantly smaller in mice transplanted with DKO compared with *Apoe*$^{-/-}$ donor bone marrow (Figure 2A; and Figure 2B, panel b versus a). Interestingly, and in agreement with the studies using whole-body DKO mice, calcification in the lesions of the aortic root was also significantly decreased in mice transplanted with *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ donor bone marrow (Figure 2C; and Figure 2B, panel d versus c and b versus a), consistent with smaller lesions in the latter mice.

En face analysis of the descending aorta indicated a trend toward lower lesions in those mice receiving bone marrow from *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ mice, although the difference failed to reach statistical significance (Figure 2D and E). However, lesion coverage in the thoracic and abdominal sections, but not the proximal sections, was significantly smaller in mice transplanted with DKO cells (Supplemental Figure V), consistent with slower lesion progression in mice receiving DKO donor cells.

Increased Macrophage Apoptosis in Lesions of DKO Mice

After 16 weeks on the Western diet, the aortic root lesions of DKO mice contained significantly more TUNEL-positive cells, often present as multicell aggregates (Figure 3A). A similar difference was seen in the bone marrow transplantation studies in which we observed a 22-fold increase in TUNEL-positive cells in lesions of *Apoe*$^{-/-}$ mice transplanted with DKO compared with *Apoe*$^{-/-}$ donor cells (Figure 3C).

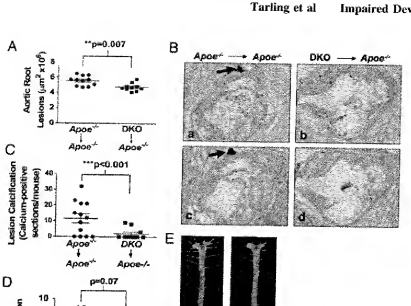

Figure 2. *Apoe*$^{-/-}$ mice lacking ABCG1 in hematopoietic cells have reduced atherosclerotic lesions. *Apoe*$^{-/-}$ mice were transplanted with bone marrow from *Apoe*$^{-/-}$ or DKO mice before being fed a Western diet for 12 weeks. All analyses were performed as described in Figure 1. A, C, and D, Lesion size (A) and calcification (C) in the aortic root sections (10 to 13 mice per group) and lesion size in the descending aorta (D) (13 to 16 mice per group) are shown, with each point representing 1 mouse. B, Representative sections from the aortic root after staining with oil red O and counterstaining with hematoxylin and fast green (a and b) or adjacent sections stained with von Kossa (c and d). Arrows identify calcium deposits. E, Representative Sudan IV–stained sections of the descending aorta. Data represent mean±SEM. $^{**}P<0.01$ and $^{***}P<0.001$.

One of the late events in apoptosis involves cleavage of the precursor form of caspase 3 to form an active protease.[29] To identify cells undergoing apoptosis within the lesions of the aortic root, frozen sections from the aortic roots of mice were immunostained with antibodies to macrophages and to the cleaved form of caspase 3. An analysis of multiple stained sections indicated that lesions of *Apoe*$^{-/-}$ mice had few active caspase 3–positive cells, whereas numerous active caspase 3–positive cells, often present as aggregates, were present in the lesions of DKO mice (Figure 3B) and in *Apoe*$^{-/-}$ mice that were the recipients of the DKO bone marrow (Figure 3D). These tissue sections also stained positive for macrophages when costained with anti-Mac3 (Figure 3B, D). An analysis of the merged Figure showed that cleaved caspase 3–positive and anti-Mac3–positive cells colocalized in the lesions of mice lacking ABCG1 (Figure 3B and D), thus identifying the apoptotic cells as macrophages. Interestingly, cleaved caspase 3– or TUNEL-positive endothelial cells were never observed in any section, suggesting that loss of ABCG1 from endothelial cells did not result in accelerated apoptosis in vivo (data not shown).

Taken together, the data from studies with whole-body DKO mice and after bone marrow transplantation demonstrate that loss of ABCG1 from hematopoietic cells alone is sufficient to slow the progression of atherosclerotic lesions. This is associated with an increase in the number of apoptotic cells in the lesions and decreased calcification within the lesion. All these changes occur by mechanisms that are independent of ApoE.

Identification of Specific Oxysterols Accumulating in *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ Macrophages

The identification of specific sterols that accumulate within macrophages in atherosclerotic lesions is complicated by the inability to obtain sufficient numbers of cells. Consequently, we performed bronchoalveolar lavage on *Apoe*$^{-/-}$ and *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ mice and recovered alveolar macrophages. Analyses of these samples using isotope dilution mass spectrometry identified a number of oxysterols, including 24-, 25-, and 27-hydroxycholesterols that accumulate in the *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ and *Abcg1*$^{-/-}$ cells compared with wild-type or *Apoe*$^{-/-}$ cells (Table). Also, compared with *Apoe*$^{-/-}$ mice, 25- and 27-hydroxycholesterol levels are significantly increased in the brain specimens of the DKO mice (Table). Therefore, the increase in the levels of these enzymatically synthesized oxysterols is not limited to macrophages.

Abcg1$^{-/-}$ Bone Marrow–Derived Macrophages Display a Proapoptotic Phenotype and an Altered Sensitivity to Oxysterols

The data of Figure 4A show that after 7 days in culture, *Abcg1*$^{-/-}$ and *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ bone marrow–derived macrophages (BMDMs) exhibited a 3- to 6-fold increase in TUNEL-positive cells, compared with wild-type or *Apoe*$^{-/-}$ cells. Although exposure of all these cells to oxLDL for 8 hours increased TUNEL staining, the highest levels of apoptosis/TUNEL staining were seen when cells lacked ABCG1 (Figure 4A), consistent with the proposal that ABCG1 is critical for limiting apoptosis in response to lipid loading. As expected, oxLDL treatment of wild-type, *Apoe*$^{-/-}$, *Abcg1*$^{-/-}$, or *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ BMDMs increased the expression of the Liver X Receptor (LXR) target genes *Abca1* and *Srebp1c* and the antiapoptotic gene *Aim* (Supplemental Figure VIA-C).

Based on the finding that specific oxysterols accumulate in alveolar macrophages and the brain specimens of *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ mice (Table), we next investigated whether cells

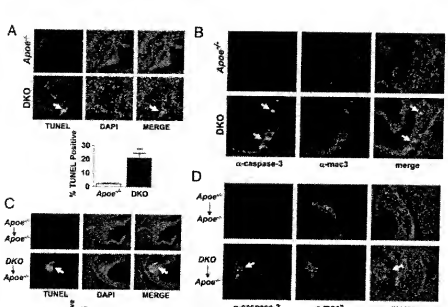

Figure 3. ABCG1 deficiency results in increased numbers of apoptotic macrophages in atherosclerotic lesions. A through D, The indicated whole-body knockout mice (A and B) or bone marrow–transplanted mice (C and D) were fed a Western diet, as described in the legends to Figure 1 and Figure 2. TUNEL- and 4′,6-diamidino-2-phenylindole–positive cells (green and blue, respectively) were determined in adjacent sections of the aortic roots (A and C) of the indicated mice. Aggregated TUNEL-positive cells are indicated by arrows. Graphs show the percentage of TUNEL-positive cells in the lesions. B and D, Adjacent frozen sections were also stained with either antibody to cleaved caspase 3 (green foci marked by arrows) or macrophages (red). The merged images are also shown (B and D). Data are representative of multiple stained sections (n=15 per section per mouse; 3 mice per genotype). Data are expressed as mean±SEM. ***$P<0.001$.

lacking ABCG1 and/or ApoE are particularly sensitive to these same oxysterols. In the absence of added oxysterols, the number of BMDMs undergoing apoptosis was 4-fold greater in *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ compared with *Apoe*$^{-/-}$ cells (Figure 4B). The addition of 10 μmol/L 7-ketocholesterol, 25-hydroxycholesterol, or 27-hydroxycholesterol increased the number of TUNEL-positive cells (Figure 4B). More important, the percentage of apoptotic cells was greatest after the addition of oxysterols to the DKO BMDMs (Figure 4B).

Table. Accumulation of Specific Oxysterols in Macrophages and Brains of Mice Lacking ABCG1

Tissue & Genotype	Cholesterol (μg/mg)	24(S)-OH-Cholesterol (ng/mg)	25-OH-Cholesterol (ng/mg)	27-OH-Cholesterol (ng/mg)
Macrophages				
WT	21.57	0.1	0.2	0.6
Abcg1$^{-/-}$	115.95	4.7	62.1	17.9
Apoe$^{-/-}$	48.78	3.6	41.9	4.1
Abcg1$^{-/-}$ *Apoe*$^{-/-}$	93.95	4.1	52.6	19.8
Brain				
Apoe$^{-/-}$	16.43±0.3	42.03±1.7	0.03±0.003	0.31±0.008
Abcg1$^{-/-}$ *Apoe*$^{-/-}$	17.17±0.6	50.06±4.3	0.07*±0.009	0.47**±0.02

Macrophages and brain sterol levels are given as μg or ng sterol/mg protein (macrophages) or /mg wet weight (brain), respectively. Macrophages were isolated from 5 mice/genotype and combined for sterol analysis. Brain sterols were determined for individual brains (6 brains/genotype) and the mean and SEM provided. *$P<0.005$; **$P<0.00025$.

The increased sensitivity of cells lacking ABCG1 to oxysterol-induced apoptosis suggested that these cells might also exhibit altered expression of genes involved in apoptosis. Consequently, we performed a PCR-based screen to identify apoptotic genes that were altered after exposure of cells to 50 μg/mL oxLDL (data not shown). Confirmation of altered gene expression came from subsequent quantitative RT-PCR analysis that showed that incubation of cells with specific oxysterols increased the expression of *Bid* and *Bok*, 2 members of the Bcl-2 proapoptotic gene family (Figure 4C and D). More important, the expression of Bid and Bok was higher in DKO or *Abcg1*$^{-/-}$ BMDMs compared with wild-type or *Apoe*$^{-/-}$ cells (Figure 4C and D).

Discussion

Herein, we report on the generation and initial characterization of *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ mice. Studies with both whole-body *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ mice and after bone marrow transplantation into *Apoe*$^{-/-}$ mice demonstrate that atherosclerotic lesion progression is reduced when mice lack ABCG1 in

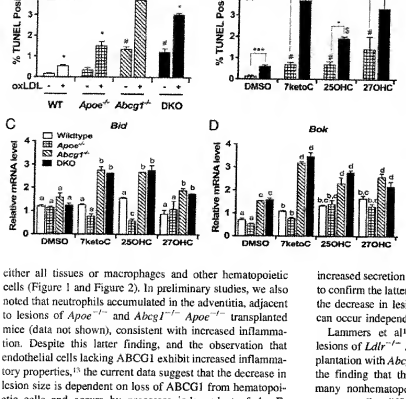

Figure 4. Macrophages lacking ABCG1 exhibit increased apoptosis in response to oxLDL or oxysterols and enhanced induction of proapoptotic genes. BMDMs in quadruplicate were differentiated in L929-conditioned media containing 10% fetal bovine serum. After 7 days, the media were replaced with media containing 0.2% BSA with or without oxLDL, 50 μg/mL (A), or the indicated oxysterol, 10 μmol/L (B–D). After 8 hours, the number of TUNEL-positive apoptotic cells (total, >1000 cells per field; 6 fields per genotype) (A and B) or the mRNA levels of *Bid* and *Bok* (C and D) were determined. Data are expressed as mean±SEM and are representative of 2 experiments. In A, * indicates significantly different from controls that were incubated with buffer ($P<0.001$); # indicates significantly different from buffer-treated WT and *Apoe*$^{-/-}$ cells ($P<0.001$). In B, # indicates significantly different from dimethylsulfoxide-(DMSO) treated *Apoe*$^{-/-}$ cells ($P<0.001$) and $ indicates significantly different from DMSO-treated DKO cells ($P<0.001$). In C and D, bars with different letters (a, b, c, d) are significantly different from one another at $P<0.001$.

either all tissues or macrophages and other hematopoietic cells (Figure 1 and Figure 2). In preliminary studies, we also noted that neutrophils accumulated in the adventitia, adjacent to lesions of *Apoe*$^{-/-}$ and *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ transplanted mice (data not shown), consistent with increased inflammation. Despite this latter finding, and the observation that endothelial cells lacking ABCG1 exhibit increased inflammatory properties,[13] the current data suggest that the decrease in lesion size is dependent on loss of ABCG1 from hematopoietic cells and occurs by processes independent of ApoE. Whether the profound decrease in calcium deposition in the atherosclerotic lesions (Figure 1 and Figure 2 and Supplemental Figure IV) is simply a consequence of the smaller lesions or of the increase in apoptotic macrophages in the lesions will require additional studies.

The importance of macrophage apoptosis in affecting early lesion development was initially reported by Arai et al[22] as a result of studies with mice lacking the antiapoptotic gene *Aim*. More important, the expression of *Aim* is largely restricted to macrophages.[22] Arai et al demonstrated a remarkable (>90%) attenuation of atherosclerotic lesions in hyperlipidemic *Aim*$^{-/-}$ *Ldlr*$^{-/-}$ mice, compared with *Aim*$^{+/+}$ *Ldlr*$^{-/-}$ mice.[22] These data suggested that increased apoptosis of macrophages limited early development and progression of atherosclerotic lesions.[22] Strikingly, in the current study, we show that cells lacking ABCG1 are more susceptible to oxysterols/induced apoptosis despite increased expression of *Aim* mRNA.

The current data extend a previous report in which it was shown that atherosclerotic lesions were decreased in *Apoe*$^{-/-}$ recipient mice after repopulation with *Abcg1*$^{-/-}$ *Apoe*$^{+/+}$ cells, compared with *Abcg1*$^{+/+}$Apoe$^{+/+}$ (wild-type) bone marrow.[16] However, the interpretation of the latter result was complicated by the fact that the expression of ApoE in the donor marrow cells is sufficient to attenuate atherosclerosis in recipient *Apoe*$^{-/-}$ mice.[20] It was further complicated by the report[18] that loss of ABCG1 from macrophages resulted in increased secretion of ApoE. Although we have not been able to confirm the latter finding, the current data demonstrate that the decrease in lesion progression after deletion of ABCG1 can occur independent of ApoE.

Lammers et al[17] recently reported that atherosclerotic lesions of *Ldlr*$^{-/-}$ *Apoe*$^{+/+}$ mice were increased after transplantation with *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ bone marrow.[17] However, the finding that the transplanted mice expressed ApoE in many nonhematopoietic cells makes comparison with the current studies difficult. This same group previously reported either no change or an increase[6,18] in lesion size after transplantation of *Abcg1*$^{-/-}$ bone marrow into hyperlipidemic *Ldlr*$^{-/-}$ mice. Whether these differences in lesion progression relate to differences in serum cholesterol levels[31] or to differences in genetic background of the mice, length of time on different diets, and the extent of the disease remains unclear.

The results of the present, and previous, work are consistent with a more important role of the ABCG1 transporter for efflux of a number of oxysterols than for efflux of cholesterol.[5,20,21,32,33] Thus, loss of this transporter leads to accumulation of 7-β-hydroxycholesterol, 7-ketocholesterol, and 24-, 25-, and 27-hydroxycholesterol as well as cholesterol. The accumulation of the side chain–oxidized oxysterols is surprising in view of their physiochemical properties, which allow them to pass biomembranes at a much higher rate than cholesterol.[34] In addition to oxysterols, desmosterol (an intermediate in the cholesterol biosynthetic pathway) has been shown to accumulate in ABCG1-deficient cells.[33] The cytotoxic and apoptotic properties of the side chain–oxidized oxysterols are well documented.[34,35] We show here that exposure of macrophages lacking ABCG1 to 7-ketocholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol leads to increased apoptosis.

In summary, our results are consistent with the possibility that at least part of the apoptotic effects of a loss of the ABCG1 transporter is due to the accumulation of oxysterols. We also

demonstrate that loss of ABCG1 from macrophages results in increased expression of the 2 proapoptotic genes *Bid* and *Bok*. Whether such changes in proapoptotic genes are sufficient to contribute to the increased apoptosis, despite an increase in the expression of the antiapoptotic gene *Aim*, is unknown.

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Disclosures

None.

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Impaired Development of Atherosclerosis in *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ Mice; Identification of Specific Oxysterols that both Accumulate in *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ Tissues and Induce Apoptosis

Supplemental Methods

Plasma Lipid and Lipoprotein Analysis

Plasma cholesterol and triglyceride, and high-density lipoprotein cholesterol (HDL-C) were determined enzymatically for individual mice as described.[1] The cholesterol lipoprotein profile was obtained after combining plasma from 6-8 or 13-16 mice/group (Supplemental Fig. 1A or 1B, respectively) and analyzing 100-200 µl on FPLC.[2]

Quantification of Atherosclerotic Lesions and Calcium Deposits

Briefly, the heart and proximal aorta were excised, washed and the apex and lower half of the ventricles removed. The remainder was embedded and frozen on dry ice in Tissue-Tek (Miles). Serial cryosections were taken through the ventricle until the appearance of the aortic valves. Serial 10 µm sections (50-60 sections per mouse), obtained from the appearance to the disappearance of the aortic valves, were collected on poly-D-lysine coated slides. Alternate sections (25-30/mouse; n=6 mice/group in Fig. 1 and n=10-13 mice/group in Fig.2) were then stained with Oil Red O and counterstained with haematoxylin and fast green prior to examination by light microscopy.[3] Atherosclerotic lesions per section were scored by determining the total intimal area using an ocular µm^2 grid.[3] The average atherosclerotic lesion area was quantified and normalized to 50 sections.

To identify mineral calcium deposition, sections consecutive to those analyzed for atherosclerotic lesions were stained by von Kossa stain.[4, 5] Positive calcium salt deposits are identified by brown-black deposits. Calcium deposits

identified by von Kossa staining were also easily identified after staining adjacent tissue sections with hematoxylin and counter staining with fast green; under these latter conditions calcium deposits give an intense purple staining.[5] Vascular calcification in the aortic lesion was identified as intense purple staining and scored semi-quantitatively as the number of positive stained sections per mouse, by an observer who was unaware of the genotypes of the mice (n=6-13 mice/group as indicated in the legends). Photomicrographs taken at high magnification are presented in supplemental Fig. 4 demonstrate that the calcium deposition is within the lesion adjacent to the medial layer.

Quantification of the lesion size/coverage in the entire descending aorta by *en face* analysis was performed as described (n=8 mice/group in Fig.1 and n=13-16 mice/group in Fig. 2).[6] Each aorta was stained with Sudan IV and three low power photomicrographs taken that correspond to the aortic arch (from the aortic root to the first intercostal), the thoracic segment (from the first intercostal to the mesenteric artery) and the abdominal segment ending at the iliac artery bifurcation. The total area and the area corresponding to the lesion (staining positive with Sudan IV) were determined in the each of the three sections and the values routinely added together to determine the percent lesion coverage. In supplemental Fig. 5 the lesion coverage in each individual segment is indicated separately.

LacZ Expression

β-galactosidase staining of the frozen sections of the aortic root was performed as previously described.[7] Briefly, hearts, lungs or spleens were removed and placed in ice-cold 4% paraformaldehyde for 4 hours. After rinsing with PBS, tissues were cryoprotected by immersing in 20% sucrose in PBS at 4^{0}C overnight. The next day hearts were snap frozen in Optimal Cutting Temperature^{TM} (OCT) embedding medium on dry ice and stored at -80^{0}C. Ten micrometer sections of the aortic root were collected on glass slides (Fisher super frost plus) and stored at -80^{0}C for future use. β-galactosidase enzymatic activity was detected by incubating slides containing sections in buffer containing

X-gal (5-bromo-4-chloro-3-indolylβ-Dgalactopyranoside) for 12-16 hours at room temperature.[7] Slides were washed in PBS/0.1% Tween-20 three times, counterstained with nuclear fast red, briefly washed with water, dehydrated and mounted using organic based media.

Immunohistochemistry

Immunohistochemical detection of cleaved caspase-3 and macrophages was carried out on 10 μM frozen sections. Sections were blocked in 5% normal goat serum in 1xPBS/0.3% Triton X-100 (blocking buffer) for 30 min. Primary antibodies were diluted (1:500) in blocking buffer and sections were incubated at 4°C overnight in a humidified chamber. Slides were rinsed 3x in 1xPBS. Cleaved caspase-3 and mac-3 were detected using species-specific rabbit polyclonal antibodies conjugated to AlexaFluor® fluorochromes (Molecular Probes), also diluted (1:500) in blocking buffer and incubated at room temperature for 1 hour. Cleaved caspase-3 was detected using goat anti-rabbit AlexaFluor® 488 (green) and mac-3 was detected using goat anti-rat AlexaFluor® 594 (red). Slides were rinsed 3x in 1xPBS before mounting on cover slips with Prolong® Gold Anti-fade (Molecular Probes).

RNA Isolation and Analysis

RNA was extracted from bone marrow derived macrophages using the Trizol/Chloroform method (Invitrogen). cDNA synthesis was performed using 1μg of DNase1-treated RNA using DNA-free kit (Ambion). Real-time quantitative PCR was performed using LightCycler 480 SYBR Green Master mix (Roche) on a Light Cycler 480 II detection system (Roche). Primer sequences are available upon request. Values were normalized to levels of the ribosomal protein 36B4 and calculated using the absolute quantification 2nd derivative max method (Roche).

TUNEL-staining

The presence of apoptotic cells was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay of paraffin-embedded tissue sections or cultured cells as previously described.[8] Five random fields from at least 3 sections of the aortic root from each of the *Apoe*$^{-/-}$ recipients receiving *Apoe*$^{-/-}$ or DKO bone marrow or from *Apoe*$^{-/-}$ or DKO mice were analyzed for apoptotic TUNEL-positive and total (DAPI-stained) cells (average n=2547)(4 mice per genotype).

Statistical Analysis

Aortic root and *en face* atherosclerotic lesion data from bone marrow transplant studies was analyzed by unpaired Student *t* test. Atherosclerotic lesion coverage in the proximal, mid and distal thirds of the descending aortas was analyzed by two-way ANOVA, with genotype of the bone marrow donor as one factor and aortic section as another. Lesion calcification and apoptosis in the atherosclerotic lesions and freshly isolated macrophages was analyzed by unpaired Student *t* test. Apoptosis in macrophages treated with oxLDL was analyzed by two-way ANOVA, with genotype of the bone marrow donor as one factor and treatment with ox-LDL as another. Results are expressed as means ±SEM. Where there was an effect of either genotype or treatment with no apparent interaction, data were further analyzed by *post hoc* Bonferroni test to determine differential effects.

Supplemental Figure Legends

Supplemental Figure I. *Apoe*$^{-/-}$ or DKO mice (A) or *Apoe*$^{-/-}$ mice transplanted with indicated bone marrow (B) were fed a western diet for 12 or 16 weeks respectively and fasted overnight. Plasma was combined, (A) 8 or (B) 13-16 mice/genotype, and analyzed by FPLC as described.[2]

Supplemental Figure II. DKO mice accumulate neutral lipid in the lung and spleen. Frozen tissue sections from *Apoe*$^{-/-}$ or DKO mice were stained for β-galactosidase (LacZ) activity, or with Oil Red O, as indicated.

Supplemental Figure III. LacZ positive cells accumulate in atherosclerotic lesions of DKO mice.

Supplemental Figure IV. Calcium deposition in the atherosclerotic lesions. *Apoe*$^{-/-}$ mice, or *Apoe*$^{-/-}$ mice transplanted with *Apoe*$^{-/-}$ marrow were fed a high fat diet for 12-16 weeks. Shown are photomicrographs of von Kossa-stained sections of the aortic root from an *Apoe*$^{-/-}$ mouse (A) and from an *Apoe*$^{-/-}$ mouse transplanted with *Apoe*$^{-/-}$ bone marrow (B). A higher magnification of the calcium deposits are shown for each, demonstrating that the calcium deposits are within the lesions and adjacent to the media.

Supplemental Figure V. Atherosclerotic lesions are decreased in the thoracic and abdominal aorta in bone marrow transplanted mice. *Apoe*$^{-/-}$ mice received bone marrow from *Apoe*$^{-/-}$ or DKO mice prior to administration of a western diet for 12 weeks. The descending aorta was stained with Sudan IV and lesions quantified in the proximal, thoracic and abdominal sections as described in Methods (n=13-16 mice/genotype). Data are expressed as mean ±SEM.

Supplemental Figure VI. Induction of LXR target genes following treatment of macrophages with oxidized LDL. Bone marrow derived macrophages were isolated from wild type, *Apoe*$^{-/-}$, *Abcg1*$^{-/-}$ or *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ mice and cultured for 8 days before addition of oxidized LDL (oxLDL) (50 μg/ml) for 8 h. RNA was isolated for quadruplicate dishes prior to RT-qPCR analysis of the indicated mRNAs. (A) * $p<0.001$, significantly different from (-) oxLDL; $^{\#}$ $p<0.05$, significantly from WT, *Apoe*$^{-/-}$ and DKO oxLDL-treated. (B) * $p<0.001$, significantly different from (-) oxLDL; $^{\#}$ $p<0.05$, significantly different from WT, *Apoe*$^{-/-}$, *Abcg1*$^{-/-}$ oxLDL-treated. Data are expressed as mean ±SEM.

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Supplemental Table I **Plasma Lipid Levels of *Apoe*$^{-/-}$ and *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ Mice**

Genotype	Triglyceride (mg/dL)	Total Cholesterol (mg/dL)	HDL (mg/dL)	Unesterified Cholesterol (mg/dL)	Free Fatty Acids (mg/dL)
Apoe$^{-/-}$	51.50 ± 1.91	1064.50 ± 86.56	17.00 ± 1.27	357.00 ± 31.51	33.50 ± 2.18
Abcg1$^{-/-}$ *Apoe*$^{-/-}$	70.00 ± 9.36	935.33 ± 39.87	15.17 ± 2.04	315.33 ± 12.79	27.83 ± 1.66

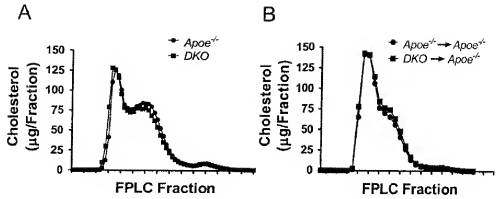
Eight week old single or DKO mice (n=8 mice/group) were fed a western diet and plasma lipids determined 16 weeks later. Data are presented as mean ±SEM.

Supplemental Table II **Plasma Lipid Levels of *Apoe*$^{-/-}$ Mice following Bone Marrow Transplantation**

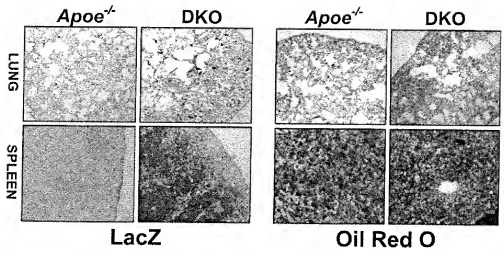
Donor Cell Genotype	Triglyceride (mg/dL)	Total Cholesterol (mg/dL)	HDL (mg/dL)	Unesterified Cholesterol (mg/dL)	Free Fatty Acids (mg/dL)
Apoe$^{-/-}$	28.13 ± 2.98	1104.75 ± 64.80	14.50 ± 0.99	324.56 ± 17.83	34.31 ± 0.80
Abcg1$^{-/-}$ *Apoe*$^{-/-}$	29.77 ± 4.24	1131.62 ± 53.29	14.23 ± 0.90	328.62 ± 18.99	36.08 ± 1.18

Eight week old *Apoe*$^{-/-}$ mice (13-16 mice/group) were transplanted with the indicated donor bone marrow. Four weeks later they were fed a western diet and plasma lipid levels were determined after 12 weeks on the diet. Data are presented as mean ±SEM.

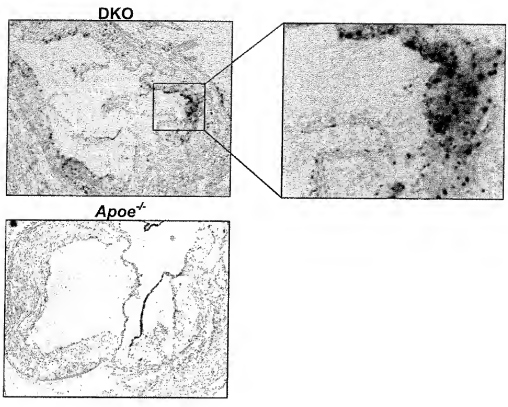
Supplemental Fig. I



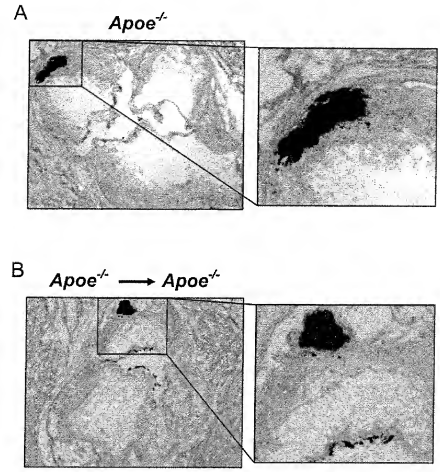
Supplemental Fig. II



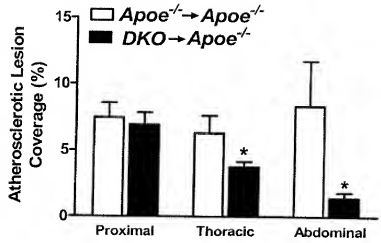
Supplemental Fig. III



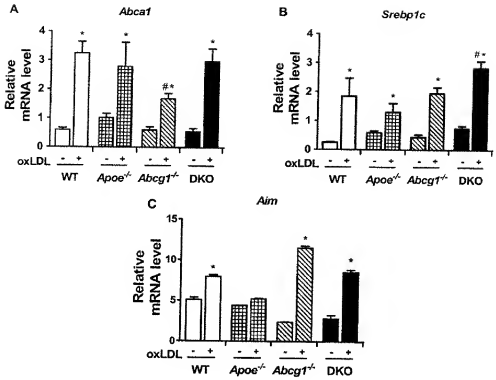
Supplemental Fig. IV



Supplemental Fig. V



Supplemental Fig. VI



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In re Brana, 34 USPQ2d 1436 (Fed. Cir. 1995)

34 USPQ2d 1436
In re Brana
U.S. Court of Appeals Federal Circuit
No. 93-1393
Decided March 30, 1995
51 F3d 1560

Headnotes

PATENTS

[1] Patentability/Validity -- Utility (▶ 115.10)

Patentability/Validity -- Specification -- Enablement (▶ 115.1105)

Application for pharmaceutical invention did not fail to disclose specific disease against which claimed compounds are useful, and thereby fail to satisfy enablement requirement of 35 USC 112, since specification, which favorably compares compounds of invention with known compounds found to be highly effective against lymphocytic leukemia tumor models, implicitly asserts that claimed compounds are also highly effective against those models, and since tumor models are cell lines representing specific lymphocytic tumors.

[2] Patentability/Validity -- Utility (▶ 115.10)

Patentability/Validity -- Specification -- Enablement (▶ 115.1105)

Patent and Trademark Office improperly rejected, for lack of utility, application claims for pharmaceutical compounds used in cancer treatment in humans, since neither nature of invention nor evidence proferred by PTO would cause one of ordinary skill in art to reasonably doubt asserted utility, and since even if utility of compounds could be reasonably questioned, evidence that compounds within scope of claims, and other structurally similar compounds, are effective as chemotherapeutic agents in animals would be sufficient to convince one skilled in art of asserted utility; absence of evidence that claimed compounds have chemotherapeutic effect in humans does not warrant contrary conclusion, since proof of alleged pharmaceutical property for compound by statistically significant tests using standard experimental animals is sufficient to establish utility.

Case History and Disposition

Appeal from the U.S. Patent and Trademark Office, Board of Patent Appeals and Interferences.

Patent application of Miguel F. Brana, Jose M.C. Berlanga, Marina M. Moset, Erich Schlick and Gerhard Keilhauer, serial no. 07/533,944, filed June 4, 1990, which is a continuation of serial no. 213,690, filed June 30, 1988. From decision upholding examiner's rejection of claims 10-13, applicants appeal.

Reversed.

Attorneys

Malcolm J. MacDonald, Herbert B. Keil, and David S. Nagy, Washington, D.C., for appellants.

Fred E. McKelvey, Solicitor, PTO; Albin F. Drost, Deputy Solicitor; Richard E. Schafer, Teddy S. Gron, Joseph G. Piccolo and Richard L. Torczon, Associate Solicitors, for appellee.

Judge

Before Plager, Lourie, and Rader, circuit judges.

Opinion Text

Opinion By:

Plager, J.

Miguel F. Brana, *et al.* (applicants), appeal the March 19, 1993 decision of the United States Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences (Board), in Appeal No. 92-1196. The Board *affirmed* the examiner's rejection of claims 10-13 of patent application Serial No. 533,944 under 35 U.S.C. Section 112 Para.1 (1988). [1]The examiner's rejection, upon which the Board relied in rendering its decision, was based specifically on a challenge to the utility of the claimed compounds and the amount of experimentation necessary to use the compounds. We conclude the Board erred, and reverse.

[1] Unless otherwise noted, all United States Code citations are to the 1988 edition.

I. BACKGROUND

On June 30, 1988, applicants filed patent application Serial No. 213,690 (the '690 application) [2] directed to 5-nitrobenzo [de]isoquinoline-1,3-dione compounds, for use as antitumor substances, having the following formula:

[2] This is a divisional of patent application Serial No. 110,871 filed October 21, 1987.

where n is 1 or 2, R^1 and R^2 are identical or different and are each hydrogen,

C1-C6-alkyl, C1-C6-hydroxyalkyl, pyrrolidinyl, morpholino, piperidinyl or piperacinyl, and R^3 and R^4 are identical or different and are each hydrogen, C1-C6-alkyl, C1-C6-acyl, C2-C7-alkoxycarbonyl, ureyl, aminocarbonyl or C2-C7-alkylaminocarbonyl. These claimed compounds differ from several prior art

benzo [de]isoquinoline-1,3-dione compounds due to the presence of a nitro group (O2N) at the 5-position and an amino or other amino group (NR ^{3}R ^{4}) at the 8-position of the isoquinoline ring.

The specification states that these non- symmetrical substitutions at the 5- and 8-positions produce compounds with "a better action and a better action spectrum as antitumor substances" than known benzo [de]isoquinolines, namely those in K.D. Paull et al., *Computer Assisted Structure- Activity Correlations, Drug Research, 34(II),* 1243-46 (1984) (Paull). Paull describes a computer-assisted evaluation of benzo [de]isoquinoline-1,3-diones and related compounds which have been screened for antitumor activity by testing their efficacy *in vivo* [3] against two specific implanted murine (i.e., utilizing mice as test subjects) lymphocytic leukemias, P388 and L1210. [4]These two *in vivo* tests are widely used by the National Cancer Institute (NCI) to measure the antitumor properties of a compound. Paull noted that one compound in particular, benzo [de]isoquinoline-1,3(2H)dione,5-amino-2(2-dimethyl-aminoethyl [sic]) (hereinafter "NSC 308847"), was found to show excellent activity against these two specific tumor models. Based on their analysis, compound NSC 308847 was selected for further studies by NCI. In addition to comparing the effectiveness of the claimed compounds with structurally similar compounds in Paull, applicants' patent specification illustrates the cytotoxicity of the claimed compounds against human tumor cells, *in vitro,* [5]and concludes that these tests "had a good action." [6]

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[3] *In vivo* means " [i]n the living body, referring to a process occurring therein." *Steadman's Medical Dictionary* 798 (25th ed. 1990). *In vitro* means " [i]n an artificial environment, referring to a process or reaction occurring therein, as in a test tube or culture media." *Id.*

[4] The analysis in Paull consisted of grouping the previously-tested compounds into groups based on common structural features and cross- referencing the various groups, in light of the success rates of the group as a whole, to determine specific compounds that may be effective in treating tumors.

[5] *See supra* note 3.

[6] The specification does not state the specific type of human tumor cells used in this test.

The examiner initially rejected applicants' claims in the '690 application as obvious under 35 U.S.C. Section 103 in light of U.S. Patent No. 4,614,820, issued to and referred to hereafter as Zee-Cheng *et al.* Zee-Cheng *et al.* discloses a benzo [de]isoquinoline compound for use as an antitumor agent with symmetrical substitutions on the 5-position and 8-position of the quinoline ring; in both positions the substitution was either an amino or nitro group. [7]Although not identical to the applicants' claimed compounds, the examiner noted the similar substitution pattern (i.e., at the same positions on the isoquinoline ring) and concluded that a mixed substitution of the invention therefore would have been obvious in view of Zee-Cheng *et al.*

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[7] The chemical compound in Zee-Cheng *et al.* is labeled a 3,6-disubstituted-1,8-naphthalimide and uses different numbering for the positions on the isoquinoline ring. The structure of this compound, however, is identical to that claimed by the applicants except for symmetrical substitutions at the 5-position and the 8-position of the isoquinoline ring. Zee-Cheng *et al.* teaches identical substitutions of amino or nitro groups while applicants claim a nitro group substitution at the 5-position and an amino group substitution at the 8-position.

In a response dated July 14, 1989, the applicants rebutted the Section 103 rejection. Applicants asserted that their mixed disubstituted compounds had unexpectedly better antitumor properties than the

symmetrically substituted compounds in Zee-Cheng *et al.* In support of this assertion applicants attached the declaration of Dr. Gerhard Keilhauer. In his declaration Dr. Keilhauer reported that his tests indicated that applicants' claimed compounds were far more effective as antitumor agents than the compounds disclosed in Zee-Cheng *et al.* when tested, *in vitro*, against two specific types of human tumor cells, HEp and HCT-29. [8]Applicants further noted that, although the differences between the compounds in Zee-Cheng *et al.* and applicants' claimed compounds were slight, there was no suggestion in the art that these improved results (over Zee-Cheng *et al.*) would have been expected. Although the applicants overcame the Section 103 rejection, the examiner nevertheless issued a final rejection, on different grounds, on September 5, 1989.

[8] HEp cells are derived from laryngeal cancer and HCT-29 cells from colon cancer.

On June 4, 1990, applicants filed a continuation application, Serial No. 533,944 (the '944 application), from the above-mentioned '690 application. Claims 10-13, the only claims remaining in the continuation application, were rejected in a final office action dated May 1, 1991. Applicants appealed the examiner's final rejection to the Board.

In his answer to the applicants' appeal brief, the examiner stated that the final rejection was based on 35 U.S.C. Section 112 Para.1. [9]The examiner first noted that the specification failed to describe any specific disease against which the claimed compounds were active. Furthermore, the examiner concluded that the prior art tests performed in Paull and the tests disclosed in the specification were not sufficient to establish a reasonable expectation that the claimed compounds had a practical utility (i.e. antitumor activity in humans). [10]

[9] The examiner's answer noted that the final rejection also could have been made under 35 U.S.C. Section 101 for failure to disclose a practical utility.

[10] The examiner subsequently filed two supplemental answers in response to arguments raised by the applicants in supplemental reply briefs.

In a decision dated March 19, 1993, the Board *affirmed* the examiner's final rejection. The three-page opinion, which lacked any additional analysis, relied entirely on the examiner's reasoning. Although noting that it also would have been proper for the examiner to reject the claims under 35 U.S.C. Section 101, the Board *affirmed* solely on the basis of the Examiner's Section 112 Para.1 rejection. This appeal followed.

II. DISCUSSION

At issue in this case is an important question of the legal constraints on patent office examination practice and policy. The question is, with regard to pharmaceutical inventions, what must the applicant prove regarding the practical utility or usefulness of the invention for which patent protection is sought. This is not a new issue; it is one which we would have thought had been settled by case law years ago. [11]We note the Commissioner has recently addressed this question in his Examiner Guidelines for Biotech Applications, see 60 Fed.Reg. 97 (1995); 49 Pat. Trademark & Copyright J. (BNA) No. 1210, at 234 (Jan. 5, 1995).

[11] *See, e.g., Cross v. Iizuka,* 753 F.2d 1040, 224 USPQ 739 (Fed.Cir. 1985); *In re Langer,* 503 F.2d 1380, 183 USPQ 288 (CCPA 1974); *In re Krimmel,* 292 F.2d 948, 130 USPQ 215 (CCPA 1961); *In re Bergel,* 292 F.2d 958, 130 USPQ 205 (CCPA 1961).

The requirement that an invention have utility is found in 35 U.S.C. Section 101: "Whoever invents . . . any new and *useful* . . . composition of matter . . . may obtain a patent therefor. . . ." (emphasis added). It is also implicit in Section 112 Para.1, which reads:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Obviously, if a claimed invention does not have utility, the specification cannot enable one to use it.

As noted, although the examiner and the Board both mentioned Section 101, and the rejection appears to be based on the issue of whether the compounds had a practical utility, a Section 101 issue, the rejection according to the Board stands on the requirements of Section 112 Para.1. It is to that provision that we address ourselves. [12] The Board gives two reasons for the rejection; [13]we will consider these in turn.

[12] This court's predecessor has determined that absence of utility can be the basis of a rejection under both 35 U.S.C. Section 101 and Section 112 Para.1. *In re Jolles,* 628 F.2d 1322, 1326 n.11, 206 USPQ 885, 889 n.11 (CCPA 1980); *In re Fouche,* 439 F.2d 1237, 1243, 169 USPQ 429, 434 (CCPA 1971) (" [I]f such compositions are in fact useless, appellant's specification cannot have taught how to use them."). Since the Board *affirmed* the examiner's rejection based solely on Section 112 Para.1, however, our review is limited only to whether the application complies with Section 112 Para.1.

[13] The Board's decision did not expressly make any independent factual determinations or legal conclusions. Rather, the Board stated that it "agree [d] with the examiner's well reasoned, well stated and fully supported by citation of relevant precedent position in every particular, and any further comment which we might add would be redundant." *Ex parte Brana et al.* , No. 92-1196 (Bd. Pat. App. & Int. March 19, 1993) at 2-3. Therefore, reference in this opinion to Board findings are actually arguments made by the examiner which have been expressly adopted by the Board.

1.

The first basis for the Board's decision was that the applicants' specification failed to disclose a specific disease against which the

claimed compounds are useful, and therefore, absent undue experimentation, one of ordinary skill in the art was precluded from using the invention. *See Hybritech Inc. v. Monoclonal Antibodies, Inc.* , 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed.Cir. 1986), cert. denied, 480 U.S. 947 (1987). In support, the Commissioner argues that the disclosed uses in the '944 application, namely the "treatment of diseases" and "antitumor substances," are similar to the nebulous disclosure found insufficient in *In re Kirk,* 376 F.2d 936, 153 USPQ 48 (CCPA 1967). This argument is not without merit.

In *Kirk* applicants claimed a new class of steroid compounds. One of the alleged utilities disclosed in the specification was that these compounds possessed "high biological activity." *Id.* at 938, 153 USPQ at 50. The specification, however, failed to disclose which biological properties made the compounds useful. Moreover, the court found that known specific uses of similar compounds did not cure this defect since there was no disclosure in the specification that the properties of the claimed compounds were the same as those of the known similar compounds. *Id.* at 942, 153 USPQ at 53. Furthermore, it was not alleged that one of skill in the art would have known of any specific uses, and therefore, the court concluded this alleged use was too obscure to enable one of skill in the art to use the claimed invention. *See also Kawai v. Metlesics,* 480 F.2d 880, 178 USPQ 158 (CCPA 1973).

[1] *Kirk* would potentially be dispositive of this case were the above-mentioned language the only assertion of utility found in the '944 application. Applicants' specification, however, also states that the claimed compounds have "a better action and a better action spectrum as antitumor substances" than known compounds, specifically those analyzed in Paull. As previously noted, *see supra* note 4, Paull grouped various benzo [de]isoquinoline-1,3-diones, which had previously been tested *in vivo* for antitumor activity against two lymphocytic leukemia tumor models (P388 and L1210), into various structural classifications and analyzed the test results of the groups (i.e. what percent of the compounds in the particular group showed success against the tumor models). Since one of the tested compounds, NSC 308847, was found to be highly effective against these two lymphocytic leukemia tumor models, [14]applicants' favorable comparison implicitly asserts that their claimed compounds are highly effective (i.e. useful) against lymphocytic leukemia. An alleged use against this particular type of cancer is much more specific than the vaguely intimated uses rejected by the courts in *Kirk* and *Kawai. See, e.g., Cross v. Iizuka,* 753 F.2d at 1048, 224 USPQ at 745 (finding the disclosed practical utility for the claimed compounds -- the inhibition of thromboxane synthetase in human or bovine platelet microsomes -- sufficiently specific to satisfy the threshold requirement in *Kirk* and *Kawai.*)

[14] Paull also found NSC 308847 to be effective against two other test models, B16 melanoma and Colon C872.

The Commissioner contends, however, that P388 and L1210 are not diseases since the only way an animal can get sick from P388 is by a direct injection of the cell line. The Commissioner therefore concludes that applicants' reference to Paull in their specification does not provide a specific disease against which the claimed compounds can be used. We disagree.

As applicants point out, the P388 and L1210 cell lines, though technically labeled tumor models, were originally derived from lymphocytic leukemias in mice. Therefore, the P388 and L1210 cell lines do represent actual specific lymphocytic tumors; these models will produce this particular disease once implanted in mice. If applicants were required to wait until an animal naturally developed this specific tumor before testing the effectiveness of a compound against the tumor *in vivo,* as would be implied from the Commissioner's argument, there would be no effective way to test compounds *in vivo* on a large scale.

We conclude that these tumor models represent a specific disease against which the claimed compounds are alleged to be effective. Accordingly, in light of the explicit reference to Paull, applicants' specification alleges a sufficiently specific use.

2.

The second basis for the Board's rejection was that, even if the specification did allege a specific use, applicants failed to prove that the claimed compounds are useful. Citing various references, [15]the Board found, and the Commissioner now argues, that the tests offered by the applicants to prove utility

were inadequate to convince one of ordinary skill in the art that the claimed compounds are useful as antitumor agents. [16]

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[15] *See* Pazdur et al., *Correlation of Murine Antitumor Models in Predicting Clinical Drug Activity in Non-Small Cell Lung Cancer: A Six Year Experience*, 3 *Proceedings Am. Soc. Clin. Oncology* 219 (1984); Martin et al., *Role of Murine Tumor Models in Cancer Research*, 46 *Cancer Research* 2189 (April 1986).

[16] As noted, this would appear to be a Section 101 issue, rather than Section 112.

This court's predecessor has stated:

> [A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of Section 112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

In re Marzocchi, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971). From this it follows that the PTO has the initial burden of challenging a presumptively correct assertion of utility in the disclosure. *Id.* at 224, 169 USPQ at 370. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility. *See In re Bundy*, 642 F.2d 430, 433, 209 USPQ 48, 51 (CCPA 1981). [17]

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[17] *See also In re Novak*, 306 F.2d 924, 928, 134 USPQ 335, 337 (CCPA 1962) (stating that it is proper for the examiner to request evidence to substantiate an asserted utility unless one with ordinary skill in the art would accept the allegations as obviously valid and correct); *In re Chilowsky*, 229 F.2d 457, 462, 108 USPQ 321, 325 (CCPA 1956) (" [W]here the mode of operation alleged can be readily understood and conforms to the known laws of physics and chemistry . . . no further evidence is required."). *But see In re Marzocchi*, 439 F.2d at 223, 169 USPQ at 369-70 ("In the field of chemistry generally there may be times when the well-known unpredictability of chemical reactions will alone be enough to create a reasonable doubt as to the accuracy of a particular broad statement put forward as enabling support for a claim. This will especially be the case where the statement is, on its face, contrary to generally accepted scientific principles.").

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[2] The PTO has not met this initial burden. The references cited by the Board, Pazdur and Martin, [18]do not question the usefulness of any compound as an antitumor agent or provide any other evidence to cause one of skill in the art to question the asserted utility of applicants' compounds. Rather, these references merely discuss the therapeutic predictive value of *in vivo* murine tests -- relevant only if applicants must prove the ultimate value in humans of their asserted utility. Likewise, we do not find that the nature of applicants' invention alone would cause one of skill in the art to reasonably doubt the asserted usefulness.

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[18] *See supra* note 15.

The purpose of treating cancer with chemical compounds does not suggest an inherently unbelievable undertaking or involve implausible scientific principles. *In re Jolles,* 628 F.2d at 1327, 206 USPQ at 890. Modern science has previously identified numerous successful chemotherapeutic agents. In addition, the prior art, specifically Zee Cheng *et al.* , discloses structurally similar compounds to those claimed by the applicants which have been proven *in vivo* to be effective as chemotherapeutic agents against various tumor models.

Taking these facts -- the nature of the invention and the PTO's proffered evidence -- into consideration we conclude that one skilled in the art would be without basis to reasonably doubt applicants' asserted utility on its face. The PTO thus has not satisfied its initial burden. Accordingly, applicants should not have been required to substantiate their presumptively correct disclosure to avoid a rejection under the first paragraph of Section 112. *See In re Marzocchi,* 439 F.2d at 224, 169 USPQ at 370.

We do not rest our decision there, however. Even if one skilled in the art would have reasonably questioned the asserted utility, i.e., even if the PTO met its initial burden thereby shifting the burden to the applicants to offer rebuttal evidence, applicants proffered sufficient evidence to convince one of skill in the art of the asserted utility. In particular, applicants provided through Dr. Kluge's declaration [19] test results showing that several compounds within the scope of the claims exhibited significant antitumor activity against the L1210 standard tumor

model *in vivo*. Such evidence alone should have been sufficient to satisfy applicants' burden.

[19] The declaration of Michael Kluge was signed and dated June 19, 1991. This declaration listed test results (i.e. antitumor activity) of the claimed compounds, *in vivo*, against L1210 tumor cells and concluded that these compounds would likely be clinically useful as anti-cancer agents. Enablement, or utility, is determined as of the application filing date. *In re Glass,* 492 F.2d 1228, 1232, 181 USPQ 31, 34 (CCPA 1974). The Kluge declaration, though dated after applicants' filing date, can be used to substantiate any doubts as to the asserted utility since this pertains to the accuracy of a statement already in the specification. *In re Marzocchi,* 439 F.2d at 224 n.4, 169 USPQ at 370 n.4. It does not render an insufficient disclosure enabling, but instead goes to prove that the disclosure was in fact enabling when filed (i.e., demonstrated utility).

The prior art further supports the conclusion that one skilled in the art would be convinced of the applicants' asserted utility. As previously mentioned, prior art -- Zee Cheng *et al.* and Paull -- disclosed structurally similar compounds which were proven *in vivo* against various tumor models to be effective as chemotherapeutic agents. Although it is true that minor changes in chemical compounds can radically alter their effects on the human body, *Kawai,* 480 F.2d at 891, 178 USPQ at 167, evidence of success in structurally similar compounds is relevant in determining whether one skilled in the art would believe an asserted utility. *See Rey-Bellet v. Engelhardt,* 493 F.2d 1380, 181 USPQ 453 (CCPA 1974); *Kawai,* 480 F.2d 880, 178 USPQ 158 .

The Commissioner counters that such *in vivo* tests in animals are only preclinical tests to determine whether a compound is suitable for processing in the second stage of testing, by which he apparently means *in vivo* testing in humans, and therefore are not reasonably predictive of the success of the claimed compounds for treating cancer in humans. [20]The Commissioner, as did the Board, confuses the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption. *See Scott v. Finney,* 34 F.3d 1058, 1063,

32 USPQ2d 1115, 1120 (Fed.Cir. 1994) ("Testing for the full safety and effectiveness of a prosthetic device is more properly left to the Food and Drug Administration (FDA). Title 35 does not demand that such human testing occur within the confines of Patent and Trademark Office (PTO) proceedings.").

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[20] We note that this discussion is relevant to the earlier discussion as well. If we were to conclude that these *in vivo* tests are insufficient to establish usefulness for the claimed compounds, that would bear on the issue of whether one skilled in the art would, in light of the structurally similar compounds in Paull and Zee Cheng *et al.* , have cause to doubt applicants' asserted usefulness for the compounds.

Our court's predecessor has determined that proof of an alleged pharmaceutical property for a compound by statistically significant tests with standard experimental animals is sufficient to establish utility. *In re Krimmel,* 292 F.2d 948, 953, 130 USPQ 215, 219 (CCPA 1961); *see also In re Bergel,* 292 F.2d 958, 130 USPQ 205 (CCPA 1961). In concluding that similar *in vivo* tests were adequate proof of utility the court in *In re Krimmel* stated:

> We hold as we do because it is our firm conviction that one who has taught the public that a compound exhibits some desirable pharmaceutical property in a standard experimental animal has made a significant and useful contribution to the art, even though it may eventually appear that the compound is without value in the treatment of humans.

Krimmel, 292 F.2d at 953, 130 USPQ at 219. Moreover, NCI apparently believes these tests are statistically significant because it has explicitly recognized both the P388 and L1210 murine tumor models as standard screening tests for determining whether new compounds may be useful as antitumor agents.

In the context of this case the Martin and Pazdur references, on which the Commissioner relies, do not convince us otherwise. Pazdur only questions the reliability of the screening tests against lung cancer; it says nothing regarding other types of tumors. Although the Martin reference does note that some laboratory oncologists are skeptical about the predictive value of *in vivo* murine tumor models for human therapy, Martin recognizes that these tumor models continue to contribute to an increasing human cure rate. In fact, the authors conclude that this perception (i.e. lack of predictive reliability) is not tenable in light of present information.

On the basis of animal studies, and controlled testing in a limited number of humans (referred to as Phase I testing), the Food and Drug Administration may authorize Phase II clinical studies. *See* 21 U.S.C. Section 355(i)(1); 5 C.F.R. Section 312.23 (a)(5), (a)(8) (1994). Authorization for a Phase II study means that the drug may be administered to a larger number of humans, but still under strictly supervised conditions. The purpose of the Phase II study is to determine primarily the safety of the drug when administered to a larger human population, as well as its potential efficacy under different dosage regimes. *See* 21 C.F.R. Section 312.21(b).

FDA approval, however, is not a prerequisite for finding a compound useful within the meaning of the patent laws. *Scott,* 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120. Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the

associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer.

In view of all the foregoing, we conclude that applicants' disclosure complies with the requirements of 35 U.S.C. Section 112 Para.1.

3.

The Commissioner takes this opportunity to raise the question of this court's standard of review when deciding cases on appeal from the PTO. Traditionally we have recited our standard of review to be, with regard to questions of law, that review is without deference to the views of the Agency, *In re Donald son,* 16 F.3d 1189, 1192, 29 USPQ2d 1845, 1848 (Fed.Cir. 1994) (in banc), *In re Caveney,* 761 F.2d 671, 674, 226 USPQ 1, 3 (Fed.Cir. 1985), and with regard to questions of fact, we defer to the Agency unless its findings are "clearly erroneous." *See, e.g., In re Baxter Travenol Labs,* 952 F.2d 388, 21 USPQ2d 1281 (Fed.Cir. 1991); *In re Woodruff,* 919 F.2d 1575, 16 USPQ2d 1934 (Fed.Cir. 1990); *In re De Blauwe,* 736 F.2d 699, 222 USPQ 191 (Fed.Cir. 1984).

With regard to judgment calls, those questions that fall " [s]omewhere near the middle of the fact-law spectrum," this court has recognized "the falseness of the fact-law dichotomy, since the determination at issue, involving as it does the application of a general legal standard to particular facts, is probably most realistically described as neither of fact nor law, but mixed." *Campbell v. Merit Systems Protection Board,* 27 F.3d 1560, 1565 (Fed.Cir. 1994). When these questions of judgment are before us, whether we defer, and the extent to which we defer, turns on the nature of the case and the nature of the judgment. *Id.* ("Characterization therefore must follow from an *a priori* decision as to whether deferring . . . is sound judicial policy. We would be less than candid to suggest otherwise.").

The Commissioner contends that the appropriate standard of review for this court regarding questions of law, of fact, and mixed questions of law and fact, coming to us from the PTO is found in the Administrative Procedure Act (APA) at 5 U.S.C. Section 706. The standard set out there is that " [t]he reviewing court shall . . . hold unlawful and set aside agency action, findings, and conclusions found to be -- (A) arbitrary, capricious, an abuse of discretion, or otherwise not in accordance with law; . . . (E) unsupported by substantial evidence. . . ." The Commissioner is of the view that the stated standard we now use, which is the traditional standard of review for matters coming from a trial court, is not appropriate for decisions coming from an agency with presumed expertise in the subject area, and is not in accord with law. [21]

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[21] Congress enacted the Administrative Procedure Act (APA) on June 11, 1946. *See* 1 *Kenneth Culp Davis, Administrative Law Treatise,* Section 1:7 (2d ed. 1978). The APA sets forth a framework for administrative agency procedure and provides judicial review for persons adversely affected by final agency actions. Chapter 7, codified at 5 U.S.C. Sections 701-706, contains the APA judicial review provisions, including the standard of review provision quoted above.

Applicants argue that by custom and tradition, recognized by the law of this court, the standard of review we have applied, even though inconsistent with the standard set forth in the APA, nevertheless is a permissible standard. In our consideration of this issue, there is a reality check: would it matter to the outcome in a given case which formulation of the standard a court articulates in arriving at its decision? The answer no doubt must be that, even though in some cases it might not matter, in others it would, otherwise the lengthy debates about the meaning of these formulations and the circumstances in which they apply would be unnecessary.

A preliminary question, then, is whether this is one of those cases in which a difference in the standard of review would make a difference in the outcome. The ultimate issue is whether the Board correctly applied the Section 112 Para.1 enablement mandate and its implicit requirement of practical utility, or perhaps more accurately the underlying requirement of Section 101, to the facts of this case. As we have

explained, the issue breaks down into two subsidiary issues: (1) whether a person of ordinary skill in the art would conclude that the applicants had sufficiently described particular diseases addressed by the invention, and (2) whether the Patent Act supports a requirement that makes human testing a prerequisite to patentability under the circumstances of this case.

The first subsidiary issue, whether the application adequately described particular diseases, calls for a judgment about what the various representations and discussions contained in the patent application's specification would say to a person of ordinary skill in

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the art. We have considered that question carefully, and, for the reasons we explained above in some detail, we conclude that the Board's judgment on this question was erroneous. Our conclusion rests on our understanding of what a person skilled in the art would gather from the various art cited, and from the statements in the application itself. We consider the Board's error to be sufficiently clear that it is reversible whether viewed as clear error or as resulting in an arbitrary and capricious decision.

The second subsidiary issue, whether human testing is a prerequisite to patentability, is a pure question of law: what does the practical utility requirement mean in a case of this kind. Under either our traditional standard or under the APA standard no deference is owed the Agency on a question of law, and none was accorded.

If the question concerning the standard of review, raised by the Commissioner, is to be addressed meaningfully, it must arise in a case in which the decision will turn on that question, and, recognizing this, the parties fully brief the issue. This is not that case. We conclude that it is not necessary to the disposition of this case to address the question raised by the Commissioner; accordingly, we decline the invitation to do so.

III. CONCLUSION

The Board erred in affirming the examiner's rejection under 35 U.S.C. Section 112 Para.1. The decision is *reversed. REVERSED* .

- End of Case -

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Ex parte JEFFERSON J. GREGORY, ROBERT G. BRUNS, DEAN R. CIROTTA, THOMAS K. ROGERS III, and CHARLES L. PAMPLIN III

Appeal 2008-005266
Application 10/360,208
Technology Center 1600

Decided:[1] June 22, 2009

Before DONALD E. ADAMS, DEMETRA J. MILLS, and LORA M. GREEN, *Administrative Patent Judges*.

GREEN, *Administrative Patent Judge*.

DECISION ON APPEAL

This is a decision on appeal under 35 U.S.C. § 134 from the Examiner's final rejection of claims 1, 8-10, 16-18, and 21. We have jurisdiction under 35 U.S.C. § 6(b).

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[1] The two-month time period for filing an appeal or commencing a civil action, as recited in 37 C.F.R. § 1.304, begins to run from the decided date shown on this page of the decision. The time period does not run from the Mail Date (paper delivery) or Notification Date (electronic delivery).

STATEMENT OF THE CASE

The claims are directed to an oral trimethobenzamide formulation that may be used to treat and control nausea and/or vomiting. Claim 1 is representative of the claims on appeal, and reads as follows:

1. An oral trimethobenzamide composition for treating and controlling nausea and/or vomiting comprising 300mg trimethobenzamide hydrochloride and a suitable pharmaceutical excipient, wherein said oral trimethylbenzamide composition is at least about as effective as a 200mg intramuscular (I.M.) trimethobenzamide HCL injectable formulation in treating and controlling nausea and/or vomiting.

The Examiner relies on the following references:

Howard C. Ansel et al., "*Pharmaceutical Dosage Forms and Drug Delivery Systems*", 7th ed. 125, (1999).

Trimethobenzamide Hydrochloride Injection and Capsules, Federal Register Notice, 44(6): 2017 (1979).

We affirm.

ISSUES

The Examiner concludes that claims 1, 8-10, 16-18, and 21 would have been obvious over the combination of the FDA Federal Register notice issued in 1979 and Ansel.

Appellants contend that the ordinary artisan reading the FDA directive would have reformulated their 100 mg and 250 mg capsules to 200 mg and 400 mg, respectively, and thus the FDA directive teaches away from a 300

mg oral formulation. Appellants contend further that the patentability of claim 1 is supported by objective evidence of non-obviousness.

Thus, the issues on appeal are:

Have Appellants demonstrated that the Examiner erred in concluding that the claims on appeal are rendered obvious by the combination of the FDA Federal register notice issued in 1979 and Ansel;

And have Appellants demonstrated that, even if a prima facie case has been established, that the Examiner erred in not considering objective evidence of non-obviousness?

FINDINGS OF FACT

FF1 "Trimethobenzamide hydrochloride is a prescription drug that has been available in the market since the 1960s." (Spec. 2.) "Even though trimethobenzamide hydrochloride has been widely available for many years, the only routes and dosage forms that have been approved by the FDA are: 100 mg and 250 mg capsules; 100 mg and 200 mg suppositories; and 100 mg/ml in 2-ml ampules and prefilled syringes and in 20-ml vials as injectables. The injectable form is intended for intramuscular administration only; it is not recommended for intravenous use." (*Id.* at 3.)

FF2 In 1979, the FDA published a notice stating that trimethobenzamide capsules containing 100 mg or 250 mg of the drug were "not approximately bioequivalent to a 200-milligram intramuscular dose and do not achieve levels necessary to effectively treat or control nausea and vomiting." (*Id.* at 5.) Thus, the FDA stated that 100 mg and 250 mg capsules must be reformulated into 200 mg and 400 mg capsules "to achieve approximate

bioequivalence to a 200-milligram intramuscular dose." (*Id.*) The Specification discloses that "[n]otwithstanding this FDA notice, which was published more than 23 years ago, there is no oral trimethobenzamide dose available today which is approximately bioequivalent to a 200-milligram intramuscular dose or which achieves plasma levels effective to control nausea and vomiting." (*Id.*)

FF3 According to the Specification, "[q]uite amazingly, it has been discovered that an oral dose of about 300 mg of trimethobenzamide is uniquely approximately bioequivalent to a 200 mg intramuscular (I.M.) trimethobenzamide HCl injectable formulation, whereas an oral dose of about 400 milligrams of trimethobenzamide is not." (*Id.* at 6.)

FF4 The Specification teaches further that "it has been discovered, quite unexpectedly, that the bioequivalency (PK) parameters of an oral dose of about 400 mg of trimethobenzamide are uniquely approximately at least about 20% greater than the corresponding bioequivalency (PK) parameters for a 200 mg intramuscular (I.M.) trimethobenzamide HCl injectable formulation." (*Id.* at 6-7.)

FF5 The Examiner rejects claims 1, 8-10, 16-18, and 21 under 35 U.S.C. § 103(a) as being obvious over the combination of FDA Federal register notice issued in 1979 (Applicants' admission in the Specification) and Ansel (Ans. 3). As Appellants do not argue the claims separately, we focus our analysis on claim 1, and claims 8-10, 16-18, and 21 stand or fall with that claim. 37 C.F.R. § 41.37(c)(1)(vii).

FF6 According to the Examiner:

> Applicants admit on pages 5-6 of the instant specification that a FDA advised public that trimethobenzamide capsules

> comprising 100 mg and 250 mg drug should be reformulated to 200 and 400 mg respectively because the bioavailability of the 100 and 250 mg dosages are not approximately equivalent to the 200 mg IM injection formulations containing the drug. Thus, by applicants' own admission the need to improve the oral dosage formulations of trimethobenzamide has been recognized.

(Ans. 3.)

FF7 The notice issued by the FDA "reclassifies trimethobenzamide and hydrochloride injection and capsules to effective for certain indications and to lacking substantial evidence of effectiveness for their other less-than-effective indications," and "states that to obtain effective plasma levels for these drug products, a dosage of 200 milligrams intramuscularly or 400 milligrams orally is required, and that as part of the marketing conditions for the capsule dosage form, the capsules, now containing 100 milligrams or 250 milligrams must be reformulated to 200 milligrams or 400 milligrams respectively." 44 Fed. Reg. 2017 (1979).

FF8 The notice further states that:

> The relative bioavailability or extent of absorption of the capsule in the two studies was 50-62 percent of that of the intramuscular injection. As the oral route of administration produced blood levels which were approximately half of the levels produced by the intramuscular route, the oral route should be approximately two times the intramuscular dose.

Id. at 2019.

FF9 Thus, the following statement was added to the Action section:

> ORAL AND PARENTAL TRIMETHOBENZAMIDE ARE NOT BIOEQUIVALENT, AN ORAL DOSE OF 400 MILLIGRAMS OF TRIMETHOBENZAMIDE YIELDS

> PLASMA LEVELS APPROXIMATELY EQUAL TO A 200-MILLIGRAM INTRAMUSCULAR DOSE. The systemic bioavailability of orally administered trimethobenzamide is about 60 percent of the bioavailability of intramuscularly administered drug, possibly because of slow absorption and rapid liver metabolism (first pass effect). This difference is manifested as diminished peak blood levels and a diminished area under the plasma concentration curve following oral, as compared to parenteral administration.

Id.

FF10 Ansel is cited by the Examiner for its discussion of various routes of drug administration, and that "drugs administered orally are destroyed or inactivated in the GI tract or are poorly absorbed to provide a satisfactory response and that the parental administration requires smaller doses of drugs." (Ans. 3.)

FF11 The Examiner concludes:

> Thus, it is evident from the teachings of Ansel as well as the FDA notice that the injection formulations require smaller doses than oral route of administration. Therefore, it would have been obvious for one of an ordinary skill in the art at the time of the instant invention was made to optimize the amounts of trimethobenzamide in an oral formulations, based on the suggestion of FDA that the 100 and 250 mg doses to be increased to 200 and 400 mg respectively because it is well established (from the teachings of Ansel) that the parenteral formulations require smaller doses than the corresponding oral formulations and that in order to achieve the same bioavailability (with oral and parenteral), one requires higher dosages of drug in an oral formulation. Accordingly, one of an ordinary skill in the art would have been motivated to optimize the amounts of oral trimethobenzamide (higher than 250) with an expectation to achieve the optimum bioavailability desired.

(*Id.* at 3-4.)

FF12 Appellants have not supplemented the record with any evidence of non-obviousness beyond what is in the FDA notice.

PRINCIPLES OF LAW

The question of obviousness is resolved on the basis of underlying factual determinations including: (1) the scope and content of the prior art; (2) the level of ordinary skill in the art; (3) the differences between the claimed invention and the prior art; and (4) secondary considerations of nonobviousness, if any. *Graham v. John Deere Co.*, 383 U.S. 1, 17 (1966).

In *KSR Int'l v. Teleflex Inc.*, 550 U.S. 398, 415 (2007), the Supreme Court rejected a rigid application of a teaching-suggestion-motivation test in the obviousness determination. The Court emphasized that "the [obviousness] analysis need not seek out precise teachings directed to the specific subject matter of the challenged claim, for a court can take account of the inferences and creative steps that a person of ordinary skill in the art would employ." *Id.* at 418. Thus, an "[e]xpress suggestion to substitute one equivalent for another need not be present to render such substitution obvious." *In re Fout*, 675 F.2d 297, 301 (CCPA 1982).

Further,

> [i]f a person of ordinary skill can implement a predictable variation, § 103 likely bars its patentability. For the same reason, if a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond his or her skill.

KSR., 550 U.S. at 418. It is proper to "take account of the inferences and creative steps that a person of ordinary skill in the art would employ." *Id. See also id.* at 421 ("A person of ordinary skill is also a person of ordinary creativity, not an automaton.").

"In cases involving ranges . . . even a slight overlap in range establishes a *prima facie* case of obviousness." *In re Peterson*, 315 F.3d 1325, 1329 (Fed. Cir. 2003). In addition, as noted by the *Peterson*, "[t]he normal desire of scientists or artisans to improve upon what is already generally known provides the motivation to determine where in a disclosed set of percentage ranges is the optimum combination of percentages." *Id.* 1330.

The prima facie case of obviousness can be rebutted "by establishing 'that the [claimed] range is critical, generally by showing that the claimed range achieves unexpected results relative to the prior art range.'" *Id.* (alteration in original). Such a showing must be commensurate in scope with the claims. *Id.* In addition, mere improvement in properties is not always sufficient to demonstrate unexpected results. *In re Soni*, 54 F.3d 746, 751 (Fed. Cir. 1995).

The other way in which the prima facie case may be rebutted is by demonstrating that the "prior art teaches away from the claimed invention in any material respect." *Peterson*, 315 F.3d at 331. *See also In re Geisler*, 116 F.3d 1465, 1469 (Fed, Cir. 1997) (citing *in re Malagari*, 499 F.2d 1297, 1303 (CCPA 1974)).

Moreover, "[w]hen prima facie obviousness is established and evidence is submitted in rebuttal, the decision-maker must start over." *In re*

Rinehart, 531 F.2d 1048, 1052 (CCPA 1976); *In re Hedges*, 783 F.2d 1038, 1039 (Fed. Cir. 1986) ("If a prima facie case is made in the first instance, and if the applicant comes forward with reasonable rebuttal, whether buttressed by experiment, prior art references, or argument, the entire merits of the matter are to be reweighed"). Thus, all of the evidence must be considered under the *Graham* factors in reaching the obviousness determination.

In speaking about the relationship of patent law and FDA law, the Federal Circuit has noted:

> On the basis of animal studies, and controlled testing in a limited number of humans (referred to as Phase I testing), the Food and Drug Administration may authorize Phase II clinical studies. See 21 U.S.C. § 355(i)(1); 5 C.F.R. § 312.23 (a)(5), (a)(8) (1994). Authorization for a Phase II study means that the drug may be administered to a larger number of humans, but still under strictly supervised conditions. The purpose of a Phase II study is to determine primarily the safety of the drug when administered to a larger human population, as well as its potential efficacy under different dosage regimens. See 21 C.F.R. § 312.21(b). FDA approval, however, is not a prerequisite for finding a compound useful within the meaning of the patent laws. . . . . Usefulness in patent law, and in particular the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer.

In re Brana, 51 F.3d. 1560, 1568 (Fed. Cir. 1995) (citations omitted). Although the above statements were made in the context of utility and enablement, the clear inference is that FDA determinations are not controlling on patentability, which would include the obviousness determination.

ANALYSIS

Appellants argue that the "FDA recognized a need for an oral formulation of trimethobenzamide hydrochloride that would yield plasma levels approximately equivalent to a 200 mg IM injection," and the FDA required that existing oral capsules be reformulated into either 200 mg or 400 mg capsules, and that the 400 mg capsule would be equivalent to the 200 mg IM injection (App. Br. 6). Thus, Appellants assert, the ordinary artisan reading the FDA directive would have reformulated their 100 mg and 250 mg capsules to 200 mg and 400 mg, respectively, not 300 mg as claimed (*id.*). Ansel, Appellants assert, is unrelated to the claims on appeal, as it "merely makes a general statement that one may employ smaller dosages of a drug when administered parentally as opposed to orally." (*Id.*)

Appellants argue further, "the prior art taught only the use of a 400 mg oral formulation to be equivalent to the 200 mg IM injection." (*Id.* at 7.) Appellants argue further that the "prior art does not suggest a range for oral administration," and "does not suggest a formula for one to determine an equivalent oral dosage strength from an IM injection strength." (*Id.*) According to Appellants, "[w]ithout any direction in the prior art, it would

not be obvious to one skilled in the art how to determine what oral dosage strength of a particular drug would be equivalent to an injectable." (*Id.* at 8.)

Appellants note that the Examiner concludes "that ' . . . a skilled artisan would have readily calculated the equivalent dosages of oral trimethobenzamide to that of intramusculsar injection . . .,'" which Appellants contend "is absurd." (*Id.* at 9.) Appellants assert that:

> The Examiner fails to note that someone in the art did calculate what it believed to be the oral dose of trimethobenzamide that would be equivalent to the 200 mg IM dose. It was calculated by the U.S. Food and Drug Administration and it was wrong.

(*Id.*)

Appellants argue further that there is objective evidence of non-obviousness (App. Br. 8). According to Appellants:

> In this case the prior art showed there was an unmet medical need for an oral dosage strength of trimethobenzamide hydrochloride that would be equivalent to a 200 mg IM injection. The prior art taught that the oral dosage would be 400 mg.
>
> Applicants were the first to show that it is a 300 mg oral dosage that provides optimal control of nausea and vomiting. The success of Applicants invention is evidenced by the FDA approval of its 300 mg product and the commercial acceptance of the invention.

(App. Br. 8.)

Appellants' arguments have been carefully considered, but are not convincing. First, as to Appellants' argument that the prior art does not teach a range, Appellants acknowledge that oral formulation comprising 100 mg, 200 mg, 250 mg, and 400 mg have been approved by the FDA in the

past. Thus, we agree with the Examiner that a range of trimethobenzamide is known to be useful for the treatment of nausea, wherein the range extends from 100 mg to 400 mg. Although that range is not explicitly taught by the FDA notice, one of ordinary skill in the pharmaceutical arts would recognize it as such. The ordinary artisan in the pharmaceutical art, which is a heavily regulated industry, would understand that only certain dosage amounts in a range that would have been obvious under the patent laws would eventually be approved for clinical use by the FDA. The ordinary artisan understands that the showing required to demonstrate that a compound intended for clinical use is patentable under the patent statute is not as strict as the showing required by the FDA for approval for *in vivo* clinical use. *See, e.g.*, *Brana*, 51 F.3d. at 1568 ("FDA approval, however, is not a prerequisite for finding a compound useful within the meaning of the patent laws.").

As we agree with the Examiner that the art establishes a range, and as claim 1 is drawn to an amount in that range, the subject matter of claim 1 is prima facie obvious under *Peterson*. We thus determine if Appellants have rebutted that prima facie case by determining if Appellants have established that the prior art teaches away from the claimed amount in any material respect, or have established that the claimed amount is critical.

As noted by Appellants, the FDA in 1979, 24 years before Appellants' filing date, reclassified trimethobenzamide capsules, and stated that "to obtain effective plasma levels for these drug products, a dosage of 200 milligrams intramuscularly or 400 milligrams orally is required, and that as part of the marketing conditions for the capsule dosage form, the capsules,

now containing 100 milligrams or 250 milligrams must be reformulated to 200 milligrams or 400 milligrams respectively." (FF7.)

The notice stated, however, that the "relative bioavailability or extent of absorption of the capsule in the two studies was 50-62 percent of that of the intramuscular injection," and thus the FDA acknowledged that there was some variability in the data (FF8). Moreover, while noting that an oral dose of 400 mg yields plasma levels *approximately* equal to that of a 200 mg intramuscular dose, as noted by the Examiner (Ans. 6), the FDA also stated that the "systemic bioavailability of orally administered trimethobenzamide is about 60 percent of the bioavailability of intramuscularly administered drug." (FF9.) Thus, 60% of a 400 mg dose would be approximately equivalent to a 240 mg IM dose, and 60% of the claimed 300 mg dose would be approximately equivalent to a 180 mg IM dose, which is "about as effective" as a 200 mg IM dose.

Thus, given the variability reported by the FDA, that is, the fact that the FDA notice stated that the "relative bioavailability or extent of absorption of the capsule in the two studies was 50-62 percent of that of the intramuscular injection," and given that the FDA notice states that the systemic bioavailability of orally administered trimethobenzamide is *about* 60 percent of the bioavailability of intramuscularly administered drug, the ordinary artisan would have been motivated to reconsider the pharmacodynamics of trimethobenzamide in order to find other dosage amounts that may be at least about as effective as a 200mg intramuscular (I.M.) trimethobenzamide. That finding is further supported by the fact that 24 years had passed since the FDA directive, and given that the background

knowledge in the art and the technology available in the pharmaceutical industry had substantially increased in those 24 years, the ordinary artisan would have been further motivated to take another look at the pharmacodynamics of trimethobenzamide, a drug that has already been approved by the FDA for clinical use. As it is the "normal desire of scientists or artisans to improve upon what is already generally known provides the motivation to determine where in a disclosed set of percentage ranges is the optimum combination of percentages," *Peterson*, we conclude that the prior art does not teach away from the composition of claim 1 in a material way. *See In re Aller*, 220 F.2d 454, (CCPA 1955) (noting that "where the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation); *see also KSR*, 550 U.S. at 421 ("When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated technical success, it is likely the product not of innovation but of ordinary skill and common sense.").

We also conclude that Appellants have not established that the claimed amount is critical. As noted above, the FDA noted there was variability in the data, and also noted that the systemic bioavailability of orally administered trimethobenzamide is about 60 percent of the bioavailability of intramuscularly administered drug. As 60% of a 300 mg oral dose would be *approximately* equivalent to a 180 mg IM dose, we conclude that the ordinary artisan would not find it unexpected that a 300 mg

oral dose would be "a least about as effective" as a 200 mg IM dose as is required by Appellants' claim 1.

While Appellants assert that the criticality of the amount is established by the FDA approval of its 300 mg product, FDA determinations are not controlling on patentability determinations, which would include the obviousness determination. In addition, while Appellants assert that there is commercial acceptance of the invention, Appellants have provided no evidence on the record that would support commercial success, or even commercial acceptance.

CONCLUSION(S) OF LAW

We conclude that:

Appellants have not demonstrated that the Examiner erred in concluding that the claims on appeal are rendered obvious by the combination of the FDA Federal register notice issued in 1979 and Ansel; and

Appellants have not demonstrated that, even if a prima facie case has been established, the Examiner erred in not considering objective evidence of non-obviousness.

We thus affirm the rejection of claims 1, 8-10, 16-18, and 21 under 35 U.S.C. § 103(a) as being obvious over the combination of FDA Federal Register Notice and Ansel.

TIME PERIOD FOR RESPONSE

No time period for taking any subsequent action in connection with this appeal may be extended under 37 C.F.R. § 1.136(a).

AFFIRMED

lp

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Paper No. 49

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Ex parte DAVID PORUBEK,
ANIL M. KUMAR, and
CHARLES R. BREDL

Appeal No. 2001-1101
Application No. 08/932,834

ON BRIEF

Before WINTERS, WILLIAM F. SMITH, and GREEN, Administrative Patent Judges.

WINTERS, Administrative Patent Judge.

DECISION ON APPEAL

This appeal was taken from the examiner's decision rejecting claims 1, 2, 4, 6, 7, 9, 10, 12 through 16, 18 through 21, and 23 through 27, which are all of the claims remaining in the application.

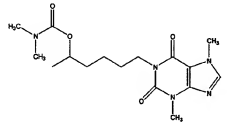
The Invention

The invention relates to compounds that form the hydroxy-substituted zanthine, lisofylline, in vivo. These compounds, or prodrugs, are said to possess a primary, characteristic benefit, viz., selective enantiomeric stability coupled with varying

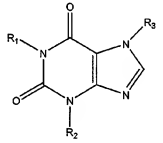
resistance to hydrolysis (Specification, paragraph bridging pages 5 and 6).

Claim 1, which is illustrative of the subject matter on appeal, reads as follows:

1. A compound having the following structure:

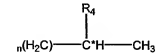


or a structure according to formula I:



I

wherein R_1 has the formula II:



R_2 and R_3 are independently $C_{(1-12)}$ alkyl, optionally, R_2 having one or two nonadjacent carbon atoms of the $C_{(1-12)}$ alkyl being replaced by an oxygen atom; and wherein:

C* is a chiral carbon atom;

n is four;

R_4 is a naturally occurring amino acid or a carbohydrate-moiety attached by an oxygen atom to the chiral carbon atom C* by an ester linkage, $-O-X-(R_5)_m$; m being two or three, depending on valence, and X being selected from the group consisting of C, P or S; wherein one R_5 is =O and any other R_5 is a member independently selected from Group Q,

said carbohydrate moiety is selected from the group consisting of glucosyl, glucosidyl, maltosyl, glucopyranosidyl, glyceraldehydyl, erythrosyl, arabinosyl, ribolucosyl, fructosyl, erythritolyl, xylosyl, lyxosyl, allosyl, altrosyl, mannosyl, mannosidyl, gulosyl, idosyl, galactosyl and talosyl, and

Group Q consists of:

hydroxyl group;

substituted or unsubstituted $C_{(3-10)}$ alkyl, $C_{(2-10)}$ alkenyl, $C_{(2-10)}$ alkynyl, $C_{(1-10)}$ alkoxyl, $C_{(1-10)}$ oxoalkyl, $C_{(1-10)}$ carboxyalkyl, $C_{(1-10)}$ hydroxyalkyl, or substituted $C_{(1-2)}$ alkyl group;

$-OR_6$, R_6 being a substituted or unsubstituted $C_{(1-10)}$ alkyl, $C_{(2-10)}$ alkenyl, $C_{(2-10)}$ alkynyl, or $C_{(1-10)}$ oxoalkyl;

substituted or unsubstituted heterocylic group, attached to X through an atom within the ring, having one or two rings, each ring containing from four to seven atoms, wherein the heteroatom(s) of said heterocyclic group is 1 or 2 nitrogens; and

substituted or unsubstituted carbocyclic group that is attached to X through a carbon atom within a ring, having one or two rings, each ring containing four to seven atoms, wherein the substituents of said substituted carbocyclic group are selected from the group consisting of amino, $C_{(2-6)}$ alkenyl, $C_{(1-6)}$ alkyl, $C_{(1-6)}$ alkoxyl, $C_{(1-6)}$ hydroxyalkyl, hydroxyl, $C_{(1-6)}$ oxoalkyl, azido, cyano, $C_{(2-6)}$ mono- or di-haloalkyl, isocyano, isothiocyano, imino, a chlorine atom, a bromine atom, a fluorine atom and an oxygen atom.

Prior Art

In rejecting the appealed claims under 35 U.S.C. § 112, first and second paragraphs, the examiner does not rely on any prior art references (Examiner's Answer, Paper No. 43, Section (9)).

The Rejections

Claims 1, 2, 4, 6, 7, 9, 10, 12, 13, 15, 16, 18 through 21, and 23 through 27 stand rejected under 35 U.S.C. § 112, second paragraph, as not particularly pointing out and distinctly claiming the subject matter which applicants regard as their invention.

Claims 1, 2, 4, 6, 7, 9, 10, 12 through 16, 18 through 21, and 23 through 27 stand rejected under 35 U.S.C. § 112, first paragraph, as based on a specification which does not adequately teach any person skilled in the art how to use the claimed invention.

Deliberations

Our deliberations in this matter have included evaluation and review of the following materials: (1) the instant specification, including Figures 1 through 10 and all of the claims on appeal; (2) the amended Appeal Brief (Paper No. 42); (3) the Examiner's Answer (Paper No. 43); and (4) the Paradise Declaration, filed under the provisions of 37 CFR § 1.132, executed June 17, 1999 (Paper No. 32).

On consideration of the record, including the above-listed materials, we affirm the examiner's rejection of claims 1, 2, 4, 6, 7, 9, 10, 12, 13, 15, 16, 18 through 21, and 23 through 27 under 35 U.S.C. § 112, second paragraph. We reverse the rejection of claim 14 under 35 U.S.C. § 112, first paragraph, and we do not reach the rejection of the remaining claims under that statutory provision.

Preliminary Matter

As a preliminary matter, we refer to section VII of the amended Appeal Brief

entitled "GROUPING OF THE CLAIMS." Applicants ask that we consider two groups of claims separately, stating that "[c]ompound claims 1, 2, 4, 6, 7, 9, 10, and 12-14 are patentable regardless of whether pharmaceutical composition claims 15, 18-21 and 23-27 are patentable" (Paper No. 42, Section VII). That section of the Appeal Brief, however, makes little sense. First, applicants have not set forth a grouping of claims "for each ground of rejection which appellant contests" as required by 37 CFR § 1.192(c)(7) (1999). Second, applicants have omitted claim 16 from their grouping of claims. Third, applicants' statement to the contrary, notwithstanding, claims 20, 21, and 23 through 27 are drawn to compounds, not pharmaceutical compositions.

Nonetheless, applicants' error in grouping claims for purposes of this appeal may be viewed as "harmless error" in view of our disposition of each ground of rejection discussed infra.

35 U.S.C. § 112, Second Paragraph

As stated in In re Zletz, 893 F.2d 319, 321-22, 13 USPQ2d 1320, 1322 (Fed. Cir. 1989): "An essential purpose of patent examination is to fashion claims that are precise, clear, correct, and unambiguous. Only in this way can uncertainties of claim scope be removed, as much as possible, during the administrative process." In the

Answer (Paper No. 43), the examiner points to several aspects of the appealed claims which are incorrect, indefinite, ambiguous, or simply make no sense. We shall not belabor the record on this point, because we agree substantially with the examiner's analysis. For reasons ably set forth by the examiner in the Answer, we affirm the rejection of claims 1, 2, 4, 6, 7, 9, 10, 12, 13, 15, 16, 18 through 21, and 23 through 27 under 35 U.S.C. § 112, second paragraph, as not particularly pointing out and distinctly claiming the subject matter which applicants regard as their invention.

We here note that the examiner rejected all claims in the application, except claim 14, under 35 U.S.C. § 112, second paragraph. Our affirmance of that rejection constitutes a disposition of the appeal with respect to all claims except claim 14.

35 U.S.C. § 112, First Paragraph

The remaining issue is whether the examiner erred in rejecting all of the appealed claims under 35 U.S.C. § 112, first paragraph, as based on a specification which does not adequately teach any person skilled in the art how to use the claimed invention. Under the circumstances, however, we shall not reach the merits of the "how to use" rejection with respect to claims 1, 2, 4, 6, 7, 9, 10, 12, 13, 15, 16, 18 through 21, and 23 through 27. Again, those claims are indefinite within the meaning of 35 U.S.C. § 112, second paragraph; they are not precise, clear, correct, and unambiguous. We think it imperative to understand the metes and bounds of the claims before proceeding to a resolution of an examiner's rejection under 35 U.S.C. § 112, first paragraph. Cf. In re Steele, 305 F.2d 859, 862, 134 USPQ 292, 295 (CCPA 1962)(Before deciding a rejection under 35 U.S.C. § 103, "it is essential to know what the claims do in fact

cover").

We now turn to a consideration of claim 14, cast in Markush format and reciting, in the alternative, 15 prodrugs of lisofylline. The examiner argues that it would require undue experimentation "to get lisofylline to actually work" (Paper No. 43, page 7, line 7). It follows, according to the examiner, that applicants' specification does not teach any person skilled in the art how to use the prodrugs recited in claim 14 without undue experimentation. In other words, the examiner's argument centers on lisofylline. If persons skilled in the art know how to use lisofylline, within the meaning of 35 U.S.C. § 112, first paragraph, the examiner would not deny that such persons would also know how to use the 15 prodrugs recited in claim 14.

It is uncontroverted on this record that "lisofylline is the subject [of] FDA-sanctioned Phase II and Phase III clinical trials." See the Paradise Declaration, filed under the provisions of 37 CFR § 1.132, executed June 17, 1999, paragraph 3 and Appendix E. As stated in In re Brana, 51 F.3d 1560, 1568, 34 USPQ2d 1436, 1442-43 (Fed. Cir. 1995), in the context of a PTO rejection under 35 U.S.C. § 112, first paragraph,

> Were we to require Phase II testing [FDA-sanctioned Phase II trials] in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer.

A fortiori, that lisofylline is the subject of FDA-sanctioned Phase II and Phase III clinical trials establishes, on this record, that any person skilled in the art knows how to use lisofylline within the meaning of 35 U.S.C. § 112, first paragraph. As stated in the Paradise Declaration, paragraph 3., "[m]erely getting such trials approved require [sic]

substantial indications of therapeutic efficacy." Accordingly, the premise of the examiner's rejection is incorrect and the rejection cannot be sustained.

Further, the examiner argues that applicants' specification does not provide useful daily dosage information (Paper No. 43, page 10, last full paragraph; and paragraph bridging pages 10 and 11). We would agree that there is room for improvement in applicants' description of a dosage regimen at page 9, first full paragraph of the specification. That passage, standing alone, is somewhat unclear. This does not, however, end the inquiry. Rather, the specification must be considered in its entirety taking into account the level of skill in the art. The following passage appears in the specification, page 9, second full paragraph:

> While dosage values will vary, therapeutic compounds of the invention may be administered to a human subject requiring such treatment as an effective oral dose of about 50 mg to about 5000 mg per day, depending upon the weight of the patient. For any particular subject, specific dosage regimens should be adjusted to the individual's need and to the professional judgment of the person administering or supervising the administration of the inventive compounds.

In our judgment, the above-quoted passage adequately conveys to any person skilled in the art useful daily dosage information for the claimed compounds.

The examiner's rejection of claim 14 under 35 U.S.C. § 112, first paragraph, is reversed.

Conclusion

In conclusion, for reasons set forth in the body of this opinion, we sustain the examiner's rejection of claims 1, 2, 4, 6, 7, 9, 10, 12, 13, 15, 16, 18 through 21, and 23 through 27 under 35 U.S.C. § 112, second paragraph.

We do not reach the merits of the "how to use" rejection under 35 U.S.C. § 112, first paragraph, with respect to claims 1, 2, 4, 6, 7, 9, 10, 12, 13, 15, 16, 18 through 21, and 23 through 27.

We do not sustain the examiner's rejection of claim 14 under 35 U.S.C. § 112, first paragraph.

The examiner's decision is affirmed-in-part.

No time period for taking any subsequent action in connection with this appeal may be extended under 37 CFR § 1.136(a).

AFFIRMED-IN-PART

Sherman D. Winters
Administrative Patent Judge

William F. Smith
Administrative Patent Judge

Lora M. Green
Administrative Patent Judge

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